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RESEARCH ARTICLE

The cost of muscle power production: muscle oxygen consumption per unit work increases at low temperatures in *Xenopus laevis*

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ABSTRACT

Metabolic energy (ATP) supply to muscle is essential to support activity and behaviour. It is expected, therefore, that there is strong selection to maximise muscle power output for a given rate of ATP use. However, the viscosity and stiffness of muscle increases with a decrease in temperature, which means that more ATP may be required to achieve a given work output. Here, we tested the hypothesis that ATP use increases at lower temperatures for a given power output in *Xenopus laevis.* To account for temperature variation at different time scales, we considered the interaction between acclimation for 4 weeks (to 15 or 25°C) and acute exposure to these temperatures. Cold-acclimated frogs had greater sprint speed at 15°C than warm-acclimated animals. However, acclimation temperature did not affect isolated gastrocnemius muscle biomechanics. Isolated muscle produced greater tetanus force, and faster isometric force generation and relaxation, and generated more work loop power at 25°C than at 15°C acute test temperature. Oxygen consumption of isolated muscle at rest did not change with test temperature, but oxygen consumption while muscle was performing work was significantly higher at 15°C than at 25°C, regardless of acclimation conditions. Muscle therefore consumed significantly more oxygen at 15°C for a given work output than at 25°C, and plastic responses did not modify this thermodynamic effect. The metabolic cost of muscle performance and activity therefore increased with a decrease in temperature. To maintain activity across a range of temperature, animals must increase ATP production or face an allocation trade-off at lower temperatures. Our data demonstrate the potential energetic benefits of warming up muscle before activity, which is seen in diverse groups of animals such as bees, which warm flight muscle before take-off, and humans performing warm ups before exercise.

KEY WORDS: Muscle performance, Locomotion, Metabolic cost, Temperature, Thermal acclimation

INTRODUCTION

Metabolic energy (ATP) supply to muscles is essential to support the normal functioning of animals in their ecological context. Long distance movement during migration (Kvist et al., 2001) or foraging (Killen et al., 2007) is constrained by access to reliable food sources to permit sufficient ATP production for muscular activity. Additionally, behavioural interactions between conspecifics are sustained by the locomotory system and incur high energetic costs (Briffa and Sneddon, 2007). Hence, success in aggressive or competitive behavioural interactions may be proportional to the capacity of cells to supply sufficient ATP for muscle performance. Similarly, in human sporting events, ATP supply determines exercise performance levels, particularly among top athletes (Jones et al., 2010).

The relationship between ATP use and muscle power output is therefore an essential determinant of ecological success across a broad spectrum of contexts. It could be expected that there is strong selection to maximise muscle power output for a given rate of ATP use, and the assumption is often made that this relationship is more or less constant, at least within populations or species (Maynard-Smith, 1994; Alexander, 1997; Santillán, 1999; Irschick and Garland, 2001). If, however, the relationship between ATP use and power output changed in response to environmental changes, the relationship between metabolic cost and the resultant benefits, in terms of movement and behaviour, would be variable.

Variation in environmental temperature affects both locomotor and muscle performance (Garland et al., 1990; James, 2013). However, it is as yet unresolved whether temperature alters the energetics of muscle performing work. It is possible that the relationship between ATP use and power output can change with temperature. The resistance of skeletal muscle to length changes comprises viscous and elastic components of the sarcomere that are independent from crossbridge formation (De Tombe and ter Keurs 1992; Fukuda et al., 2005; Mutungi and Ranatunga, 1998; Granzier and Wang, 1993). This passive tension decreases with increasing temperature because muscle becomes less viscous (Mutungi and Ranatunga, 1998). Hence, if the passive tension is great enough to affect force production (De Tombe and ter Keurs, 1992), it may cause a thermal dependence of the qualitative relationship between ATP use and muscle power output. In other words, colder muscle may require greater rates of ATP hydrolysis to achieve a given power output compared with the same muscle at a higher temperature.

Hence, our aim was to determine the relationship between isolated muscle power output and oxygen consumption in response to chronic and acute temperature changes in *Xenopus laevis* Daudin 1802. Specifically, we tested the hypotheses that with a decrease in muscle temperature the metabolic energy required to achieve a given power output increases. Alternatively, temperature may have the same effect on ATP use and muscle power output by its thermodynamic effect on protein activities so that both decrease with decreasing temperature, but the ratio between power and oxygen consumption remains constant. A corollary of the latter hypothesis is that acclimation to chronic temperature change may elicit a compensatory response so that animals will at least partially offset acute thermodynamic effects on swimming and muscle performance.

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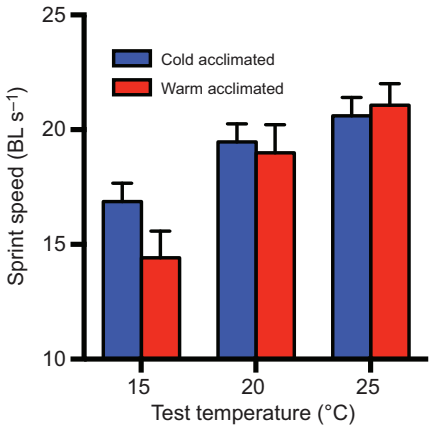

Fig. 1. Sprint speed of *Xenopus laevis* acclimated to cold (15°C) and warm (25°C) temperatures for 4 weeks. Swimming performance [body lengths (BL) s^{-1}] was measured at 15, 20 and 25°C acute test temperatures, and there was a significant interaction between acclimation and test temperature (P<0.03). N=10 for each acclimation group.

RESULTS

Swimming performance

There was a significant interaction between acclimation treatment and test temperature for frog swimming performance ($F_{2,17}$=4.36, P<0.03), and frogs from the cold acclimation treatment performed better at 15°C (Fig. 1).

Isometric mechanics of isolated gastrocnemius muscle

Isometric tetanus stress was greater at 25°C than at 15°C ($F_{1,14}$=90.74, P<0.0001) with no effect of acclimation temperature (both main effect and interaction $F_{1,14}$<0.3, P>0.55; Fig. 2A). Isometric muscle force generation (time to half peak tetanus; $F_{1,14}$=126.26, P<0.0001; Fig. 2B) and relaxation (time from last stimulus to half tetanus relaxation; $F_{1,14}$=40.20, P<0.0001; Fig. 2C) times were significantly longer at 15°C than at 25°C, and there were no effects of acclimation (main effects and interactions all $F_{1,14}$<1.2, P>0.3).

Work loop performance of isolated gastrocnemius muscle

Muscle power output was significantly greater at 25°C than at 15°C ($F_{1,14}$=70.87, P<0.0001), but there was no effect of acclimation treatment nor an interaction (both $F_{1,13}$<0.80, P>0.39; Fig. 3A). The decline in work produced at work loop 40, which is an indicator of muscle fatigue, did not differ between acclimation treatments ($F_{1,14}$=1.59, P=0.23) or test temperatures ($F_{1,14}$=3.40, P=0.086; acclimation × test temperature interaction $F_{1,14}$=0.10, P=0.92).

Oxygen consumption of gastrocnemius during rest and work loop performance

The raw data trace (Fig. 4A) shows a typical pattern of oxygen consumption, which increases rapidly between rest and activity (during work loop performance). Oxygen consumption of muscle at rest did not change significantly with test temperature ($F_{1,14}$=1.12, P=0.31) or acclimation treatment ($F_{1,14}$=0.87, P=0.37), and there was no interaction ($F_{1,14}$=0.21, P=0.66; Fig. 4B). However, maximum oxygen consumption during work loop performance of isolated muscle was significantly higher at 15°C than at 25°C ($F_{1,14}$=5.87, P<0.03; Fig. 4C), but there was no effect of acclimation nor an interaction (both $F_{1,14}$<1.2, P>0.28).

Integrating the results from the work loop power output and oxygen consumption measurements, we show that the amount of oxygen used per joule of net work output was significantly greater at 15°C than at 25°C ($F_{1,14}$=27.05, P<0.0001; Fig. 5), and that there was no effect of acclimation treatment nor an interaction (both $F_{1,14}$<1.53, P>0.24).

DISCUSSION

We have shown that the ATP required by *Xenopus* muscle to achieve a given work output increases with decreasing temperature, thereby accepting the hypothesis stated in the Introduction. Importantly, this response is not plastic, indicating that it reflects an intrinsic quality of the muscle structure that is not modulated by differential expression of genes and proteins as a result of reversible acclimation. We would predict, therefore, that developmental processes too cannot modulate the relationship between ATP use and power output. Cooler more viscous or stiffer muscle requires greater application of force to stretch, thereby reducing the net work per length change cycle and contributing to the reduction in net power output when compared with warmer muscle with less resistance (Bishop, 2003; Noonan et al., 1993). This means that the metabolic cost of muscle performance changes in animals that experience variation in body, or muscle, temperature. Importantly, this temperature dependence of muscle performance is independent from thermodynamic effects on protein function. The thermal sensitivity of muscle function and of other physiological processes is thought to be caused by thermodynamically induced decreases in protein activities at cool temperatures, and by damage to proteins and membranes at very high temperatures (James, 2013; Tattersall et al., 2012). Our data show that there is an additional dimension to the thermal dependence of muscle function that consists of thermodynamic effects on the physical properties of the muscle.

During activity and exercise, 90% of ATP consumption is by working muscle (van Beek et al., 2011). The post-exercise recovery period or oxygen debt is directly related to the intensity of exercise (Svendsen et al., 2010). There are obvious advantages to reducing

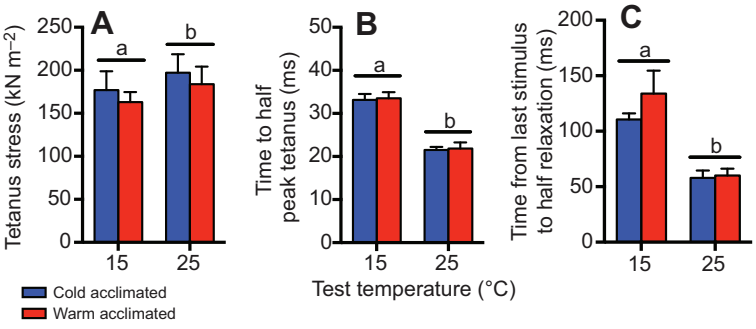

Fig. 2. Isometric mechanics of gastrocnemius muscle from cold (15°C)- and warm (25°C)-acclimated *X. laevis* measured at 15 and 25°C acute test temperatures. Tetanus stress (A) was significantly greater at 25°C test temperature than at 15°C regardless of acclimation treatment (significant differences between test temperatures are indicated by horizontal bars with different letters). Similarly, time to half peak tetanus (B) and time from last stimulus to half tetanus relaxation (C) were significantly shorter at 25°C compared with 15°C test temperature regardless of acclimation treatment. N=8 for each acclimation group.

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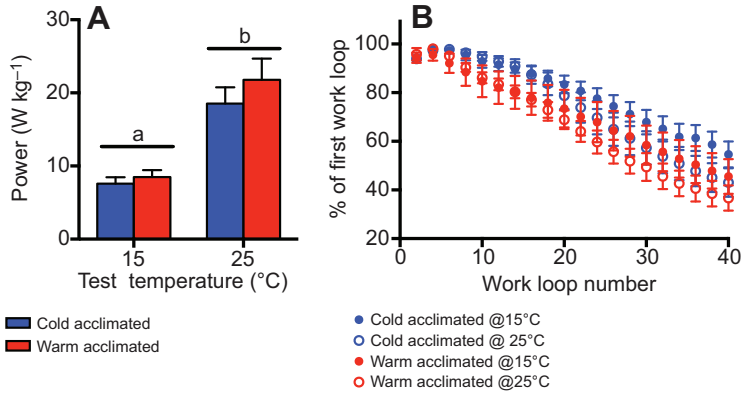

Fig. 3. Work loop power output and fatigue resistance. (A) The mean work loop power output per length change cycle, per kilogram muscle mass, of isolated gastrocnemius muscle from *X. laevis* was significantly greater at 25°C than at 15°C acute test temperature in frogs from both cold (15°C) and warm (25°C) acclimation treatments. Differences between test temperatures are indicated by horizontal bars with different letters. (B) Muscle fatigue, estimated as the decline of work (J) produced per work loop (plotted as the percentage of the work produced at the first work loop per muscle preparation), did not differ between treatments over 40 work loops. *N*=8 for each acclimation group.

the recovery period after activity by decreasing the amount of ATP used for a given activity via optimising muscle temperature. For example, animals can resume activity more quickly and are therefore better able to respond to external threats. Additionally, fatigue resistance may increase with more efficient use of ATP. Muscle fatigue is determined to a large extent by sarcoplasmic reticulum calcium depletion (Allen et al., 2008). Calcium released from the sarcoplasmic reticulum following excitation binds to troponin, thereby facilitating myosin–actin crossbridge formation and muscle force generation. Muscle relaxation is achieved by resequestering calcium into the sarcoplasmic reticulum by endo-sarcoplasmic reticulum calcium ATPase (SERCA) (Berchtold et al., 2000). Hence, both force generation and relaxation require ATP, and the activity of SERCA in particular is associated with muscle fatigue resistance (James et al., 2011).

Muscle stiffness (negative work) is determined by both the number of attached crossbridges and the viscosity of the muscle (Sugi and Tsuchiya, 1988; Mutungi and Ranatunga, 1998). The mechanical efficiency of crossbridges – that is, the ratio between power output and enthalpy output – remains constant with changes in temperature, at least in relatively fast fibre-type mouse muscle (EDL) (Barclay et al., 2010). If this were also the case for *Xenopus* muscle, then temperature-dependent changes in mechanical efficiency cannot explain the increased oxygen consumption per unit work at low temperatures that we observed. The most parsimonious explanation for the increased ATP use at low temperatures is that there is a greater number of ATP-consuming crossbridges, or greater ATP use by existing crossbridges, to achieve the same force output. However, tetanus force, which depends on the number of attached myosin–actin crossbridges and the force produced by each (Syme and Tonks, 2004), decreased at low temperature in our *Xenopus*. This decrease in tetanus stress as well as in power output at low temperature indicates that any increase in crossbridge attachment, and related increase in ATP consumption, was insufficient to compensate for the increased muscle viscosity at low temperatures even following thermal acclimation.

The slower muscle force generation and relaxation times at low temperature were most likely caused by negative thermodynamic effects on proteins involved in excitation–contraction coupling (e.g. dihydropyridine and ryanodine receptors) and relaxation (SERCA) (Berchtold et al., 2000). Colder muscle also generates force less rapidly and often produces lower peak force (James, 2013; James et al., 2011), which is an additional explanation for the reduced tetanus stress and power production at low temperatures.

Daily and seasonal variations in body temperature are particularly pronounced in ectotherms. The implications of the current findings are that muscle-powered behaviour and movement become more efficient at particular times of day or at different seasons. Many ectotherms thermoregulate behaviourally by selecting thermally suitable microhabitats to let body temperatures change towards the operative temperatures of the environment (Hertz et al., 1993; Seebacher, 2000). The rate of heat transfer is modified physiologically by changes in blood flow that can accelerate heating and retard cooling (Seebacher and Grigg, 2001). The main benefits of thermoregulation lie in reaching suitable body temperatures for organs and the nervous system to function properly. Our data indicate that rapid changes in muscle perfusion, particularly when cool animals enter a heating environment (Seebacher and Franklin, 2007), are important to facilitate the efficiency of muscle function and thereby locomotion. Some insects such as bees perform rapid contractions of their flight muscles before take-off. These contractions increase flight muscle temperature (Kovac et al., 2010) and, as we show here, will increase the energetic efficiency of flight. Hence, many ectotherms warm their muscles before movement and activity. Muscle activity and animal movement are possible at lower temperature, but would require a greater investment of ATP. These

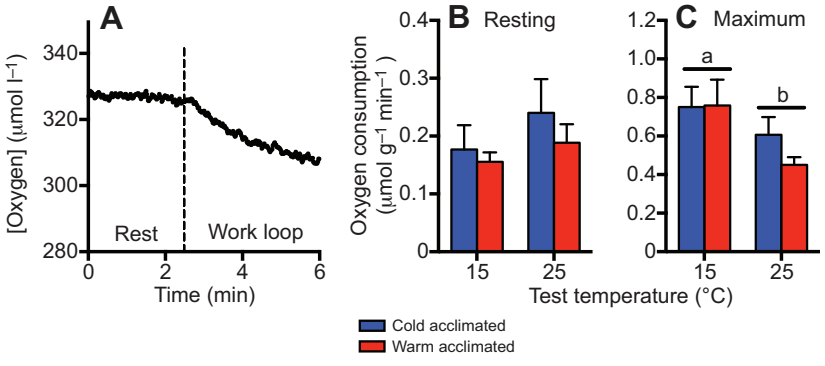

Fig. 4. Oxygen consumption by isolated gastrocnemius muscle. (A) The raw data trace shows the decline in oxygen concentration of muscle at rest (left of the dashed vertical line) and during work loop performance (right of the dashed line). (B) There was no difference in oxygen consumption of *X. laevis* isolated gastrocnemius muscle at rest between acclimation treatments (cold: 15°C; warm: 25°C). (C) However, during work loop performance, isolated muscle consumed significantly more oxygen at 15°C than at 25°C acute test temperature (indicated by horizontal bars with different letters) regardless of acclimation treatment. *N*=8 for each acclimation group.

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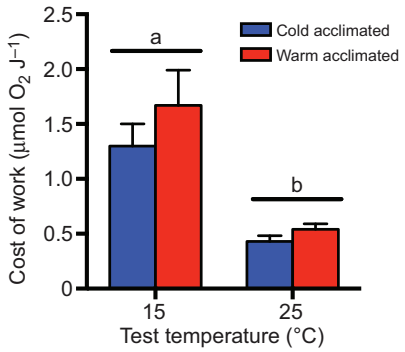

Fig. 5. The metabolic cost of work. The amount of oxygen consumed per joule of work produced (μmol O_2 J^{-1}) was significantly greater at 15°C than at 25°C acute test temperature (indicated by horizontal bars with different letters) regardless of acclimation treatment.

relationships are somewhat counterintuitive because ATP use is assumed to increase with increasing temperature, particularly in ectotherms (Dickson et al., 2002). The important finding here is that while ATP use may increase at higher temperatures, it also becomes more efficient.

At a seasonal time scale, many ectotherms acclimate locomotor performance and metabolism to compensate for the thermodynamic effect of longer term changes in temperature (Guderley, 2004; Johnston and Temple, 2002). Interestingly, our data imply that under cold conditions there should be a net increase in ATP production if muscle function and locomotor performance are to be maintained across a temperature range. Hence, for thermal acclimation to fully compensate for, say, winter conditions, it is not sufficient to maintain metabolic scope or enzyme activities at the same level as during summer; instead, there has to be an increase above summer rates so that muscle performance can remain constant across seasons. Alternatively, there may be a relative increase in the ATP allocated to muscle myosin ATPase or SERCA activity during winter. Hence, if the capacity for metabolic cold acclimation is limited, as it is likely to be (Seebacher et al., 2013), there may be an allocation trade-off (Angilletta et al., 2003). Interestingly, burst speed was higher at the lower 15°C test temperature in our *Xenopus* acclimated to low temperatures, but this was not paralleled by acclimation in muscle force production, power output or oxygen consumption. It is worth noting, however, that at lower acclimation and test temperatures (10°C), the mechanics of isolated *Xenopus* muscle differed between acclimation treatments (Wilson et al., 2002), which indicates that the muscle responds to extreme thermal conditions. A likely explanation for the differences in response between swimming and isolated muscle performance is that short bursts of locomotion rely on creatine kinase dynamics to supply ATP and are therefore independent from oxygen consumption, at least in the short term (Gray et al., 2006; Wüst et al., 2013). Additionally, it is possible that burst performance may rely more on the excitation of the muscle rather than on muscle contraction–relaxation dynamics (Robin and Allard, 2012) so that it is more dependent on neural signal transmission than on muscle function per se.

Even endotherms show considerable body temperature fluctuations (Glanville et al., 2012), and peripheral muscles in particular can be several degrees cooler than core temperatures (Robergs et al., 1991; Noonan et al., 1993). Hence, the energetic efficiency of muscle power production and locomotion will change daily and seasonally. It may be speculated from our data that, as in bees and other ectotherms, the advantage of warm-ups before exercise is to increase the energetic efficiency of muscle performance.

MATERIALS AND METHODS

Animals and swimming performance

African clawed frogs, *X. laevis* (*N*=20; mean mass ± s.e.=9.84±0.57 g; mean snout–urostyle length ± s.e.=4.29±0.11 cm), were obtained from the University of Warwick (Coventry, UK), where they were bred and reared under identical conditions; hence, differences in developmental conditions would not affect the experimental outcomes. Morphological measurements for each frog were recorded using Mitutoyo calipers (±0.01 mm; Japan). Frogs were kept in plastic tanks (645×423×276 mm; three to four frogs per tank) at 20°C at Coventry University for 2 weeks to habituate to their new surroundings. Animals were kept on a 12 h:12 h light:dark cycle and fed bloodworms daily. After 2 weeks, the temperature in the tanks was changed gradually over 3 days to reach acclimation temperatures of either 15 or 25°C (*N*=10 frogs each). Animals were kept at their acclimation temperature for 4 weeks before experiments were started, which is sufficient to induce acclimation responses in amphibians (Rogers et al., 2004).

After 4 weeks of acclimation, sprint swimming velocity was measured at 15, 20 and 25°C acute test temperatures in each frog (*N*=10 per acclimation treatment), with at least 24 h between swimming trials. Measurements of swimming performance followed published protocols (Wilson et al., 2002). Frogs were placed into shallow plastic trays (400×350 mm with a water depth of 50 mm) and startled by gently tapping their urostyle with a wire probe. The ensuing startle response resulted in an escape response consisting of several power strokes. We filmed at least three escape responses for each temperature for each individual with a camera (Casio Exilim EX F1 camera filming at 60 frames s^{-1}) and analysed the video files in Tracker Video Analysis and Modeling Tool software (Open Source Physics, www.opensourcephysics.org). We used the fastest velocity achieved at each temperature for each individual during the repeated escape responses in the analysis of sprint performance.

Isolated muscle mechanics

The frogs were killed by pithing and transection of the spinal cord in accordance with British Home Office Animals (Scientific Procedures) Act 1986, Schedule 1. The gastrocnemius muscle was removed from the right hindleg and used for the muscle performance experiments. The gastrocnemius muscle is a major locomotory muscle in frogs and is therefore suitable to test the hypotheses proposed here. All procedures were based on those previously described (James et al., 2012). Dissection was performed in oxygenated chilled (3–5°C) Ringer solution with the following composition (in mmol l^{-1}): NaCl, 115; KCl, 2.5; Na_2HPO_4, 2.15; NaH_2PO_4, 0.85; glucose, 10.0; and $CaCl_2$, 1.8; pH 7.4 at 20°C prior to oxygenation. A piece of bone was left attached to each tendon of the gastrocnemius muscle. The mean (±s.e.) mass of the preparation was 0.13±0.0068 g and the length was 14.06±0.0068 mm. We used methods described elsewhere (van der Laarse et al., 2005) to ascertain that limits to oxygen diffusion did not constrain oxygen consumption of muscle preparations.

Isometric studies (*N*=8 per acclimation treatment) were used to determine the twitch and tetanus kinetics of isolated gastrocnemius muscle. The bone at one end of the muscle preparation was clamped via a crocodile clip to a strain gauge (UF1, Pioden Controls Ltd, Canterbury, Kent, UK), and the bone at the other end was clamped via a crocodile clip to a motor arm (V201, Ling Dynamics Systems, Royston, Hertfordshire, UK) attached to an LVDT (Linear Variable Displacement Transformer, DFG 5.0, Solartron Metrology, Bognor Regis, Sussex, UK). The LVDT was used to monitor the length changes delivered to the muscle preparation. The whole of the muscle, tendon and bone preparation was then allowed to equilibrate within the bath at either 15 or 25°C for 10 min in circulating, oxygenated (95% O_2; 5% CO_2) frog Ringer solution. The muscle preparation was then held at constant length and square wave stimuli of 160 mA and 2 ms duration were delivered via two parallel platinum wire electrodes to generate a series of twitches. Stimulus amplitude (voltage) and muscle length were adjusted to determine the stimulation parameters and muscle length corresponding to maximal

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isometric twitch force. An isometric tetanus force response was elicited by subjecting the muscle to a 200 ms train of electrical stimulation. Stimulation frequency was altered (95 to 120 Hz), for each subsequent tetanus, to determine maximal tetanus force. Time to half peak tetanus force and time from last stimulus to half tetanus force relaxation were measured. A rest period of 5 min was allowed between each tetanus response. Half of the muscles from each acclimation group of frogs were first tested at 15°C, the other half of the muscles were first tested at 25°C.

The work loop technique was used to determine the power output (average of each work loop cycle) of muscles during cyclical length changes (Josephson, 1993). Unlike fixed-length isometric studies and fixed-load isotonic studies of muscle performance, the work loop technique allows measurement of muscle power output under length and activation changes that are generally more indicative of *in vivo* contractile performance (Caiozzo, 2002; James et al., 1996). In the absence of *in vivo* strain data for gastrocnemius muscle in *X. laevis*, each muscle preparation (N=8 per acclimation treatment) was subjected to a set of four sinusoidal length changes symmetrical around the length found to generate maximal twitch force. Previous research on *Bufo marinus* (Gillis and Biewener, 2000) suggests that sinusoidal length changes are likely to represent a simplification of *in vivo* strain patterns; however, they should provide a reasonable approximation of muscle performance. The muscle was stimulated using the stimulation amplitude and stimulation frequency found to yield maximal isometric force. Electrical stimulation and length changes were controlled via a data acquisition board (KUSB3116, Keithley Instruments, Cleveland, OH, USA) and a custom-designed program developed with TestPoint software (CEC TestPoint version 7, Measurement Computing, Norton, MA, USA). Muscle force was plotted against muscle length for each cycle to generate a work loop, the area of which equated to the net work produced by the muscle during the cycle of length change (Josephson, 1993). Instantaneous power output was calculated for every data point in each work loop (2000 data points per work loop) by multiplying instantaneous velocity by instantaneous force. These instantaneous power output values were then averaged to generate an average net power output for each work loop cycle. The cycle frequency of length change was altered between 2 and 8 Hz to determine the cycle frequency for maximal power output for each individual at each temperature. Muscle strain was kept at 0.11 at each cycle frequency, where a strain of 0.11 represents a length change of ±5.5% of resting muscle length, 11% peak to peak. Every 5 min, the muscle was subjected to a further set of four work loop cycles with length change cycle frequency, stimulation duration and stimulation phase parameters being altered in between each set until maximum net work was achieved at each cycle frequency and maximal power output had been determined at each test temperature. At 15°C, power output was typically maximal at a length change cycle frequency of 3 Hz; at 25°C, this value usually increased to 7 Hz.

On completion of the maximal power output determination (burst muscle performance test) at the initial acute test temperature, the test temperature of the Ringer solution bathing the muscle was altered to the other test temperature (15 or 25°C) over 10–20 min, allowing at least a further 10 min for the muscle to equilibrate to the new test temperature. The above isometric and work loop studies were then repeated at the new test temperature.

On completion of the maximal power output determination at the second test temperature, the muscle was subjected to a short, sustained high intensity (endurance) test whereby 50 work loops were delivered to the muscle whilst oxygen consumption was recorded. During the endurance test, length change cycles were delivered at a cycle frequency of 2 Hz when at 15°C or at 5 Hz when at 25°C. The stimulation delivered during the endurance test was at half the stimulation frequency found to generate maximal isometric tetanus force for that muscle at that temperature. After the endurance test the temperature of the Ringer solution bathing the muscle was altered back to the initial test temperature over 10–20 min, allowing at least a further 10 min for the muscle to equilibrate to the new test temperature. The above isometric and work loop studies, including the endurance test, were then repeated at the new test temperature. A set of control sinusoidal length change and stimulation parameters were imposed on the muscle every three to five sets of work loops, when the muscle was at the initial and final (third) common test temperature, to monitor variation in the muscle's ability to produce power/force over the time course of the experiment. Any variation in power (average power per cycle) was found to be due to a matching change in ability to produce force. On average, the net mean muscle power output per cycle decreased by 8.7% over the time course of each experiment. Therefore, the power produced by each preparation at each temperature was corrected to the control run at the initial test temperature that yielded the highest power output (average power per cycle), assuming that alterations in power-generating ability were linear over time between the control runs delivered at the first and final test temperatures.

At the end of the isometric and work loop experiments, the bones and tendons were removed and each muscle was blotted on absorbent paper to remove excess Ringer solution. Wet muscle mass was determined to the nearest 0.1 mg using an electronic balance (Mettler-Toledo B204-S, Greifensee, Switzerland). Mean muscle cross-sectional area was calculated from muscle length and mass assuming a density of 1060 kg m^{-3} (Méndez and Keys, 1960). Maximum isometric muscle stress (kN m^{-2}) at each test temperature was then calculated as maximum tetanus force divided by mean cross-sectional area. Maximum normalised muscle power output (W kg^{-1}) at each test temperature was calculated as average power output per length change cycle divided by wet muscle mass.

Isolated muscle oxygen consumption

To measure oxygen consumption of isolated muscle (N=8 per acclimation treatment) at rest and during prolonged work loop performance, we used a plastic covering to seal the Perspex bath that contained the isolated muscle during work loop measurements. A section of the plastic covering contained a fast-responding fluorescent oxygen sensor (Pst3, PreSens, Regensburg, Germany) that was submerged in the Ringer solution ~2–3 mm above the isolated muscle. The sensor was attached to a custom-made support, which formed part of the chamber seal and which allowed us to mount a fibreoptic probe to monitor oxygen content of the chamber in real-time. The probe was attached to an oxygen meter (both PreSens) connected to a laptop computer. During measurements of oxygen consumption, we stopped the flow of aerated Ringer solution by clamping the piping into and out of the chamber, and recorded oxygen concentration every second. We measured oxygen consumption of muscle at rest for 2–5 min before starting work loops. We also ran preliminary tests to ensure that there was no oxygen consumption in the chamber without the muscle but with the electrodes delivering stimuli to the chamber.

Statistical analysis

Sprint swimming velocity, muscle oxygen consumption rates, twitch and tetanus stress, time to half peak tetanus, time from last stimulus to half tetanus relaxation, normalised muscle power output, and oxygen consumed per joule of power output were analysed by analysis of variance with acclimation temperature as a fixed factor and test temperature as a repeated measure; we used Pillai's trace as the test statistic to determine significance of the repeated measure. We estimated muscle fatigue by calculating the decline of work produced over 40 work loops as a percentage of the work produced at the first work loop of each preparation. We compared the percentage work produced (arcsin-transformed data) (Quinn and Keough, 2004) between treatments at work loop 35 using acclimation treatment as a fixed factor and test temperature as a repeated measure. We tested for the homogeneity of the data using Levene's test, and all data fulfilled this assumption.

Competing interests

The authors declare no competing financial interests.

Author contributions

F.S. and R.S.J. conceived and designed the experiments, J.A.T. conducted the experiments, F.S. and R.S.J. analysed the data, F.S. wrote the manuscript, R.S.J. and J.A.T. edited the manuscript.

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