

Effects of temperature on contractile properties of skinned muscle fibers from three toad species

IAN A. JOHNSTON AND TODD T. GLEESON

Department of Environmental, Population, and Organismic Biology, University of Colorado, Boulder, Colorado 80309

JOHNSTON, IAN A., AND TODD T. GLEESON. *Effects of temperature on contractile properties of skinned muscle fibers from three toad species*. Am. J. Physiol. 252 (Regulatory Integrative Comp. Physiol. 21): R371–R375, 1987.—Single fast fibers were isolated from the iliofibularis muscles of three species of toad with different thermal minima for active locomotion: 8°C, American toad, *Bufo americanus*; 15°C, Rocky Mountain toad, *Bufo woodhousei woodhousei*; 22°C, Cane toad, *Bufo marinus*. All experiments were carried out during the summer. Fibers were chemically skinned and maximum isometric tension and unloaded contraction velocity were determined at a series of temperatures between 0 and 35°C. At 25–30°C, isometric tension development has a low temperature dependence ($R_{10} = 1.1$ – 1.3) and is in the range of 210–260 $\text{kN} \cdot \text{m}^{-2}$ for each of the three toads. However, at 0–10°C, absolute values of tension increase in the series (*B. americanus* > *B. woodhousei* > *B. marinus*; i.e., with increasing cold tolerance), while thermal sensitivity between 0 and 10°C is inversely related to cold tolerance. For example, at 0°C, maximum isometric tension (P_0) for the most northerly distributed species is three times higher than for the subtropical to tropical species ($P < 0.001$). R_{10} for P_0 (0–10°C) is 1.7 for *B. marinus*, 1.3 for *B. w. woodhousei*, and 1.0 for *B. americanus*. In contrast, unloaded shortening speeds were similar at any given temperature for the three species. It is concluded that adaptations in *Bufo* myosin for activity at low temperatures largely involves changes in force production.

skeletal muscle; *Bufo marinus*

FROG MYOSIN is of an unstable type (12). For example, Ferenczi et al. (10) found that even in fresh preparations the amplitude of protein-fluorescence quenching was maximal at 0.27 mol of thio-ITP/mol myosin subfragment 1, suggesting that only 30% was enzymatically active. Fish myosins are even more unstable, readily undergoing a side-by-side aggregation reaction on isolation, with a concomitant loss of adenosinetriphosphatase (ATPase) activity (8). Connell (9) suggested that these properties are characteristic of animals with relatively low body temperatures. Support for this idea comes from a study by Johnston and Walesby (16) on fish myofibrillar ATPase activity. They found that the half-life of thermal denaturation of the ATPase varied from only 1.5 min for three Antarctic species to over 500 min for a fish found living in an equatorial hot spring at 35–42°C.

Recently, we have used skinned muscle fibers to investigate the functional consequence of these different

myosin structures (17, 18). It has been found that the power-temperature relation varies according to the normal range of body temperatures experienced by a species. For example, at -1°C the maximum power output of fast fibers from an Antarctic teleost (*Chaenocephalus aceratus*) is only 50% of that for a tropical fish (*Makaira nigricans*) at 20°C. However, compared at 0°C, muscle power output for the Antarctic fish is some seven times higher than for the tropical species (18). These evolutionary differences largely involve the ability of myosins to generate force (17, 18). For particular fiber types, interspecific variations in unloaded shortening speeds (17) and isometric ATP turnover rates (1) are relatively small at any given temperature.

Although terrestrial ectotherms are rarely active at the extremely low (-2 to 5°C) temperatures experienced by polar fishes, there exists considerable variation in the body temperatures at which they show significant locomotory capacities (2). In the present study we have investigated the effects of temperature on the contractile properties of muscle from three species (genus, *Bufo*) collected from different latitudes in North America. The minimum temperature at which activity has been reported varies by 14°C between the most northerly and the most southerly distributed species (23). The aim of this study was to determine whether similar evolutionary adjustments to temperature occur in teleosts and anuran amphibians.

MATERIALS AND METHODS

Animals. American toads (*Bufo americanus*, 25–53 g), Rocky Mountain toads (*B. woodhousei woodhousei*, 92–130 g), and Cane or Giant toads (*B. marinus*, 22–330 g) were collected locally by suppliers in northern Wisconsin, Boulder, Colorado, and Sonora, Mexico, respectively. *B. americanus* is the most northerly distributed of the three species, ranging as far north as James Bay, Canada (55°N latitude). *B. americanus* and *B. woodhousei* are closely related species, can hybridize (4), and are sympatric across the central United States. Where sympatric, *B. americanus* breeds earlier and at cooler temperatures (4). The distribution of *B. marinus* is primarily South American, extends only as far north as the Rio Grande Valley of Texas, and does not overlap the distribution of the other two species (7). Minimum body temperatures of animals observed active in the field are 8°C (23), *B. americanus*; 15°C , *B. woodhousei*; and 22°C , *B. marinus*

(5). A body temperature of 15°C is lethal for the Giant toad (5). Although we do not have average field active temperatures of these toads near their collection sites, all three are reported to breed in April and May. Mean air temperatures during these months near the sites of collection average (°C, April/May) 7–12°C, Wisconsin; 11–16°C, Colorado; 21–25°C, south Texas (22). The above data and observations all suggest a range of cold tolerance from the northernmost to southernmost distributed species.

Histochemistry. All muscle fibers studies were dissected from a region of the iliofibularis muscle opposite the femoral artery and sciatic nerve, which adhere closely to the muscle along its medial surface. To confirm our use of a uniform population of fibers, histochemical determination of fiber type was made from 6- μm serial sections of the iliofibularis muscle of all three species. Myosin ATPase activity was determined in air-dried sections incubated for 1 min in neutral formaldehyde solution (phosphate buffer, pH 7.0 at 25°C) followed by a 30 min incubation in a reaction medium (pH 9.4, 37°C) described by Gleeson et al. (13). Oxidative fibers were identified in sections incubated for 1 h in a medium designed to identify fibers of high succinic dehydrogenase activity. The medium contained (mmol/l) NADH, 1; sodium succinate, 16; nitroblue tetrazolium, 1.4, in 200 mmol tris(hydroxymethyl)aminomethane buffer (pH 7.4 37°C).

Contractile properties. Single fast fiber segments (50–120 μm diameter, 2.5–3.5 mm length) were isolated under silicone oil (MS 550, BDH, Poole, England) at 0–4°C. Fibers were transferred to the apparatus using jeweler's forceps and attached between two stainless steel hooks using cynoacrylate (17). After mounting, fibers were rapidly immersed in the first of three water-jacketed baths (temperature control $\pm 0.2^\circ\text{C}$) and chemically skinned for 10–15 min in relaxing solution containing 1% Brij 58 (polyoxyethylene 20 cetyl ether). Relaxing solution contained (mmol/l) 1,4-piperazine-bis(ethanesulfonic acid) (PIPES), 25; ethyleneglycol-bis(β -aminoethylether)-*N,N*-tetraacetic acid (EGTA), 15; MgCl_2 , 6.8; ATP, 6; phosphocreatine, 27.5; 3% (wt/vol) PVP-360 (polyvinyl Pyrrolidone) and 25–50 U/ml creatine phosphokinase (pH value was adjusted to 7.2 at 20°C with KOH). (All reagents were from Sigma Chemical, St. Louis, MO.) Fibers were subsequently transferred to a second bath containing relaxing solution without Brij 58 for 3–5 min. Sarcomere length was measured by laser diffraction and set to 2.3 μm . Fiber diameter and length were measured in situ using a high-power microscope.

Activating solution contained in a third bath was made by addition of 15.3 mmol/l CaCl_2 . Preliminary experiments established that this Ca^{2+} concentration ($[\text{Ca}^{2+}]$) was sufficient to produce maximal contractions at each temperature. Free ion concentrations were calculated using an iterative computer program (21) incorporating corrections for temperature (14). The pH of activating solution was set at 7.2 at 20°C and allowed to vary freely with temperature such that pH changes closely followed that for the pK_a of PIPES ($-0.0088 \text{ U}/^\circ\text{C}$). This approximates to the $\Delta\text{pH}/\Delta\text{T}$ relationship established for the

skeletal muscle of a number of ectotherms (25). The main ionic species were at the following concentrations: pMg 2.96–3.02, pMg ATP 2.28–2.26, pCa 4.66–3.80, pH 7.37–7.12, and ionic strength 0.18–0.19 mol/l (values quoted are for 0 and 30°C, respectively). Preliminary experiments established that the $[\text{Ca}^{2+}]$ was sufficient to give maximal activations at each temperature and that maximum isometric tension (P_0) and maximal unloaded contraction velocity (V_{max}) were insensitive to changes in pH over this range.

Force was measured with a silicone beam strain gauge (AME 801, Horten, Norway) with a resonant frequency of 3 kHz including fiber and hook; sensitivity was 0.5 mN/V. Unloaded contraction velocity was determined for maximally activated fibers using the slack-test method. A servo system based on a loud-speaker coil (17) was used to give 1-ms releases sufficient to abolish tension (3–10% resting length), and the time to take up the slack was recorded using a storage oscilloscope. Following each release the fiber was reextended to its original length. Records were analyzed by drawing a line parallel to and above the zero force line by an amount equivalent to the noise level of the transducer. The time to take up slack (t_s) was measured from the point at which the force record crossed this line. At this point force was increasing rapidly, allowing a more accurate measurement of t_s . Unloaded contraction velocity was determined from the slope of the applied length change vs. the time taken to redevelop force (Fig. 1).

Temperature coefficients for tension generation (R_{10} 's) and contraction velocities (Q_{10} 's) were defined as $(P_2/P_1)^{10/T_2-T_1}$ where P_2 and P_1 are parameters at temperatures T_2 and T_1 , respectively, and $T_2 > T_1$ (3).

Statistical analyses. Measurements of P_0 and (V_{max}) were made on two to three fibers from each of four (*B. americanus*, *B. marinus*, or five *B.w. woodhousei*) toads at each temperature. Values for the different species were compared at 0°C using a one-way analysis of variance.

RESULTS

The region of the iliofibularis muscle from which fibers were taken for study was uniformly composed of fast-

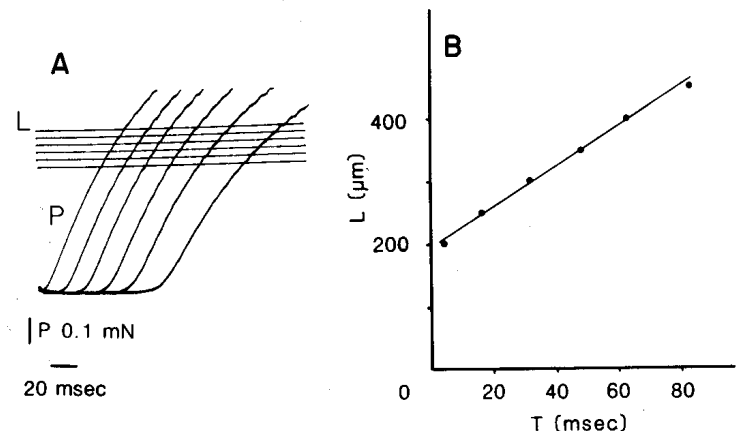


FIG. 1. Records illustrating method used to determine unloaded contraction velocity. A: length (L) and force (P) record from a single fast fiber isolated from iliofibularis muscle of *Bufo woodhousei*, showing force recovery after rapid releases of different amplitudes. Experiment at 0°C. B: plot of amplitude of release vs. time for force recovery. See text for details.

twitch fibers in all three species. Sections incubated for myosin ATPase demonstrated a small number of large-diameter fibers of low myosin ATPase activity in *B. marinus* opposite the region studied. Homologous fibers were not detected in the other two species despite slight alterations in incubation conditions. Fibers of *B. marinus* within the region of interest were lower in succinic dehydrogenase (SDH) activity than fibers in the more medial, deep region of the muscle. Fibers of *B. americanus* and *B. woodhousei* were of uniform SDH activity across the entire iliofibularis of both species.

Fibers from the three species maintained steady tensions during 2-min contractions and relaxed completely at all temperatures studied. There were significant differences between the three species in 1) the absolute tensions generated at 0–10°C and 2) the temperature dependence of P_o . Maximum tension at 0°C was correlated with minimum field active temperature (Fig. 2) and was three times higher for the most northerly (*B. americanus*) compared with the most southerly distributed toad (*B. marinus*). Intermediate values of P_o were found for the Rocky Mountain toad at 0°C (Fig. 2). A one-way analysis of variance was carried out on these data, a significant difference between groups being found, $F(2, 26) = 35.1$ ($P < 0.00001$). A Newman-Keuls test was carried out to compare the means; all pairwise comparisons were found to be significant at the 0.01 level. At 25–35°C, maximum tensions for all three species were similar and in the range 210–260 $\text{kN}\cdot\text{m}^{-2}$. P_o had a low thermal dependence over this temperature range for all the toads ($R_{10} = 1.1$ –1.3). In contrast, at low temperatures (0–10°C), R_{10} increased in the series *B. marinus* (1.7), *B. w. woodhousei* (1.2), and *B. americanus* (1.1).

In contrast to P_o , similar values for unloaded contraction velocity were obtained for fast fibers from the three toad species at any given temperature (Fig. 3). Q_{10} for

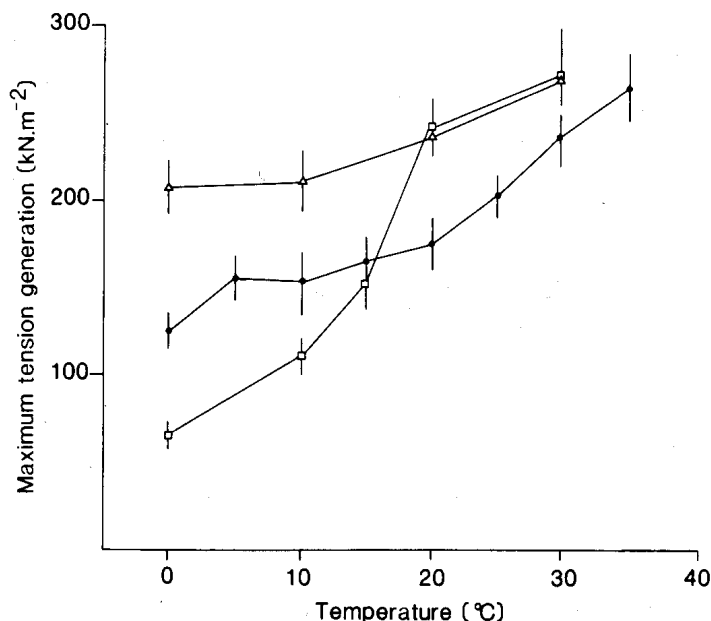


FIG. 2. Effects of temperature on maximum isometric tension generation ($\text{kN}\cdot\text{m}^{-2}$) for skinned fibers isolated from the iliofibularis muscles of 3 species of toad. Open triangles, American toad, *Bufo americanus*; filled circles, Rocky Mountain toad, *Bufo woodhousei*; open squares, Cane toad, *Bufo marinus*.

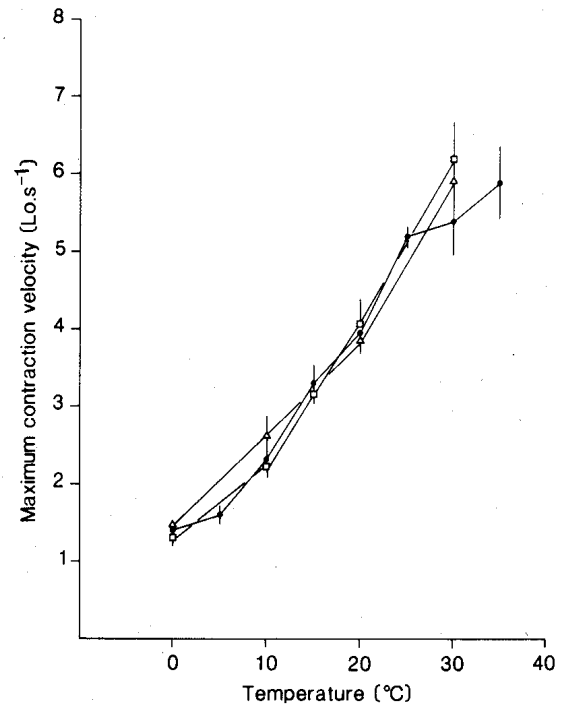


FIG. 3. Effects of temperature on unloaded contraction velocity (muscle lengths $\cdot s^{-1}$, $L_0\cdot s^{-1}$) for skinned fibers isolated from the iliofibularis muscles of 3 species of toad. Open triangles, American toad, *B. americanus*; filled circles, Rocky Mountain toad, *B. woodhousei*; and open squares, Cane toad, *B. marinus*.

V_{max} decreased slightly from 1.7–1.9 at 0–10°C to 1.4–1.5 at 20–30°C.

DISCUSSION

Changes in temperature significantly modify contractile activity in the muscle of both ectotherms and homeotherms (3). However, the effect of preferred body temperature on the relationship between temperature and various contractile parameters has rarely been considered (24, 29). The results in the present study clearly show that maximum force production, but not unloaded cross-bridge cycle time, is related to the minimum body temperature at which different species are active. These results parallel those obtained for skinned fibers isolated from the fast muscle of various teleost fish, suggesting a common underlying mechanism (17, 18).

Experiments with skinned (14, 17, 29) and live muscle fibers (6, 24) have shown that maximum isometric tension usually has a low thermal dependence ($R_{10} = 1.0$ –1.4) at normal body temperatures. However, for species with active body temperatures of 20–37°C, P_o often declines markedly as temperature is decreased outside this range. For example, Stephenson and Williams (29) could measure no force production by rat skinned muscle fibers at 0–1°C, and P_o for skinned fibers isolated from the iliofibularis muscle of the Cane toad decreased by 90% between 20 and 0°C (Ref. 29; see also Fig. 3). In contrast, P_o for northerly distributed anurans, e.g., *Bufo bufo* (whole sartorius) (6), *B. americanus* (Fig. 3), *Rana pipiens* (skinned fibers) (14), and Antarctic teleosts (skinned fibers) are generally over 200 $\text{kN}\cdot\text{m}^{-2}$ at 0°C and show only modest increases at higher temperatures (17, 18). The fast muscles of different vertebrate species generate

rather similar maximum tensions at their preferred body temperatures (200–350 kN·m⁻²) (15).

All experiments on *Bufo* were conducted during the summer months when habitat temperatures were 20–32°C for all three toads. The muscles of some cyprinidae fish have been reported to show increases in myofibrillar ATPase activity (19), unloaded contraction velocity, and isometric tension development (20) following several months of cold acclimation. In contrast, the contractile properties of whole muscles from *B. americanus* (26) and *R. pipiens* (27) appear to be fixed and not altered by temperature acclimation. Instead anuran amphibians respond to extreme cold with a behavioral strategy that involves burrowing and hibernation. The variations in the temperature-force relation reported for toad muscles in the present study are therefore likely to represent true species differences rather than different states of acclimation.

The mechanism whereby the muscles of cold-tolerant ectotherms produce high forces at low temperatures could involve an increase in the number of attached cross bridges and/or an increase in force per cross bridge. There is evidence in favor of both of these mechanisms. For example, Ford et al. (11) found that for live frog fibers instantaneous stiffness during a tetanus increased less than peak force as temperature was raised from 0 to 8°C, consistent with an increase in force generation per bridge. Stephenson and Williams (28) found that for rat skinned fibers, rigor force was 15–20 times lower at 5°C than 20°C, irrespective of whether rigor was induced independently at each temperature or at 5°C and the temperature raised. This result provides indirect evidence for the number of attached cross bridges increasing at higher temperatures. Altringham and Johnston (1) have investigated the effects of temperature on maximal force and ATPase activity in skinned fibers isolated from teleost fish. For maximal isometric contractions at –5°C, tension was 14 times greater for fibers from an Antarctic (*Notothenia neglecta*) than a tropical species (*Oreochromis niloticus*), although their isometric ATP turnover rates were similar (1). The net result is that at low temperatures, myosin from *N. neglecta* generates more force per ATP hydrolyzed.

Studies with skinned and glycerinated fibers have shown that force, unloaded contraction velocity (17, 18), and ATPase activity have different temperature dependencies (1). If, as this suggests, various steps in the cross-bridge cycle have different Q₁₀'s, then a change in temperature would result in a redistribution of the time spent in various states. For example, an increase in the rate of attachment relative to detachment would lead to an increase in the time spent in the force-generating state. If, as is generally assumed, bound time is relatively short compared with overall cross-bridge cycle time, then this could offer an explanation as to how two muscles could produce very different forces and yet have similar ATPase activities. Thus, although overall cross-bridge cycle times are similar at low temperatures in polar, temperate, and tropical ectotherms (1), there may nevertheless be substantial differences in the kinetics of intermediate steps.

We are grateful for support from National Science Foundation Grant 820 2432 to T. Gleeson.

Address for reprint requests: I. A. Johnston, Dept. of Physiology, Gatty Marine Laboratory, University of St. Andrews, St. Andrews, Fife, KY16 8LB, Scotland.

Received 16 December 1985; accepted in final form 16 September 1986.

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