Fatigue and Recovery of Dynamic and Steady-State Performance
in Frog Skeletal Muscle

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running head: fatigue and recovery of work in frog muscle

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ABSTRACT

Muscle fatigue reflects alterations of both activation and cross-bridge function, which will have markedly different affects on steady-state versus dynamic performance. Such differences offer insight into the specific origins of fatigue, its mechanical manifestation and its consequences for animal movement. These were inferred using dynamic contractions (twitches and cyclic work as might occur during locomotion) and steady state performance with maximal, sustained activation (tetani, stiffness and isokinetic force) during fatigue and then recovery of frog (*Rana pipiens*) anterior tibialis muscle. Stiffness remained unaltered during early fatigue of force, then declined only 25% as force dropped 50%, suggesting a decline with fatigue in first the force generating ability and then the number of cross-bridges. The relationship between stiffness and force was different during fatigue and recovery, thus the number of and force per cross bridge are not intimately linked. Twitch duration increased with fatigue and then recovered, with trajectories that were remarkably similar to and linear with changes in tetanic force, perhaps belying a common mechanism. Twitch force increased and then returned to resting levels during fatigue, reflecting a slowing of activation kinetics and a decline in cross-bridge number and force. Net cyclic work fatigued to the degree of becoming negative when tetanic force had declined only 15%. Steady-state isokinetic force (i.e. shortening work) declined by 75%, while cyclic shortening work declined only 30%. Slowed activation kinetics were again responsible, augmenting cyclic shortening work but greatly augmenting lengthening work (reducing net work). Steady-state measures can thus seriously mislead regarding muscle performance in an animal during fatigue.
INTRODUCTION

Fitts (19) contrasts two definitions of fatigue: failure to maintain force during repeated stimulation versus failure to maintain the required or expected power output, and suggests that power may be a more suitable measure as it reflects both the kinetics and kinematics of muscle contraction. Due to the multifaceted nature of fatigue (e.g. 1,19,54,55) such distinctions are important, both when quantifying fatigue and in interpreting how it is manifest and the nature of its consequences. Fatigue must be defined in the context of a particular aspect of performance and the cellular processes in question (24). The basis for these arguments suggests a strategy to study fatigue and its impact on animal movement. Fatigue per se is the consequence of changes in different cellular processes which in turn affect various aspects of muscle contraction. Therefore, by contrasting changes in muscle performance/contraction that rely on different cellular processes it is possible i) to further clarify the causes of fatigue, ii) to determine their relative importance at different stages of fatigue and recovery, and iii) to understand how they impact various facets of the mechanical performance of muscle and hence its ability to power animal movement. Thompson et al. (50) used a similar approach to study the etiology of muscle fatigue in frog muscle.

Here we consider fatigue in the muscle cell, as distinct from central fatigue and failure at the neuromuscular junction. In this light, deficits in mechanical performance with fatigue are due to changes in cross-bridge activity (myofibrillar fatigue) and changes in the magnitude and kinetics of activation (failure of activation) (17). The former encompasses force per cross-bridge and the cross-bridge cycle as it impacts both force and shortening. The latter includes the calcium handling machinery and its impact on the magnitude and kinetics of the intracellular free calcium transient, troponin-calcium kinetics, the cross-bridge cycle, and ultimately the number of
working cross-bridges. MacIntosh & Rassier (31) warn that the coexistence of fatigue and potentiation will compromise the ability to quantify fatigue, and while we do not attempt to separate these effects we echo their caution in interpreting results, particularly during the early stages of fatigue.

In addition to the dichotomy of activation versus cross-bridges, differences between steady-state performance (during maximal, sustained activation) and dynamic performance (during cyclic activation and relaxation) can be particularly useful in determining what mechanisms are altered and how this impacts animal movement (4,28,46,47). With these distinctions in mind, we measured steady-state and dynamic performance of frog anterior tibialis muscle during fatigue and recovery to further understand how fatigue is caused and how it impacts muscle performance in relation to animal movement. Steady-state measures included i) maximal, isometric, tetanic force (dependent on the number of strongly attached cross-bridges and the force produced by each cross-bridge), ii) muscle stiffness during tetanic contractions (reflecting the proportion of attached cross-bridges, but see methods section for cautions in interpretation), and iii) the force produced during isokinetic shortening following maximal, isometric activation (reliant on the number of attached cross-bridges, the force they produce, and their ability to cause muscle shortening; this force is also a direct index of work done while shortening). These measures reflect cross-bridge activity, but not the time course of activation; they are useful in providing insight into the mechanisms of fatigue but they tell us less about the impact of fatigue on animal movement. Dynamic measures included i) twitch force and kinetics (which depend on the time course and magnitude of activation and cross-bridge kinetics; they will also reflect characteristics of the series compliance, which we have assumed to be passive and not impacted by fatigue, although this is not certain), and ii) work performed during cyclic,
sinusoidal length cycles and phasic activation using the work loop technique (26). These latter measures are dynamic actions that reflect both cross bridge function and the time course of activation, and thus can provide insight into the effects of fatigue on work that is available for locomotion. Further, unlike work measured using isokinetic shortening contractions, work measured using cyclic contractions can be used to describe both the work done by the muscle during shortening and also the work required to re-lengthen the muscle (as would be required of its antagonist). The energy required to lengthen muscles can be of great consequence when considering the function of a muscle in a moving animal. Cyclic work is thus likely a superior indicator of a muscle's ability to contribute to animal movement and the impact of fatigue on movement.

MATERIALS AND METHODS

muscle preparation

All procedures and animal husbandry followed approved University of Calgary animal care guidelines. Leopard frogs (*Rana pipiens*) were killed by decapitation and pithing. Small bundles (n=7, length 7.5-11.0 mm, wet mass 2.0-9.5 mg) were dissected from the anterior tibialis muscle in physiological saline (in mM: 115 NaCl, 3 KCl, 2 CaCl₂, 20 NaHCO₃, 2 NaH₂PO₄, 5 glucose, pH 7.8) on a chilled stage (5°C). To minimize potential differences between preparations in fiber type distribution, bundles were always taken from the central region of the muscle. The bundle was then placed in a chamber filled with circulating, physiological saline at room temperature (22.3 - 22.8°C) and bubbled with a 98% O₂ : 2% CO₂ gas mixture. Short segments of tendon at the ends of the muscle bundle were tied to a rigid hook and to the arm of a servo motor/force transducer (Cambridge Technology Inc., series 300 dual mode, model 350)
using 5-0 silk suture; to minimize stray compliance the tendon was pulled tight against the pins so that no length of suture extended between the two. Curare (17 mg l⁻¹) (22) was added to the saline in the chamber. Platinum, stimulating electrodes were placed along the length of the muscle on each side, and connected to a battery-driven current source gated by a Grass SD9 stimulator. The stimulator and servomotor were controlled with a computer using custom software written in LabView (National Instruments, Austin, Texas). Muscle length and force signals were recorded on the computer at 5 kHz using a 12 bit A/D card (PCI-MIO-16E-4, National Instruments) and custom software written in LabView. The servomotor had a critically damped, step response time of about 500 s. The force transducer had a resonant frequency of 830 Hz.

**establishing experimental parameters**

The muscle’s length was adjusted to remove visible slack, and then carefully measured using a microscope and calibrated ocular micrometer. The stimulus voltage was adjusted to 150% of that required to elicit a maximal, isometric twitch (1 ms stimulus pulse). The muscle’s length was then systematically altered in 0.5 mm steps until maximal, isometric, twitch force was achieved. This length was used throughout experiments. For tetanic stimulation, a train of stimulus pulses was delivered at 100 Hz for 150 ms, long enough to ensure that maximal, isometric, tetanic force (Po) was attained.

To measure muscle stiffness (Fig. 1), the muscle was stretched (step function) by 0.3% of its length during the plateau of an isometric tetanus, held for 10 ms, and then released back to the original length. Another 20 ms was allowed to elapse for force to recover before the isokinetic shortening protocol described below began. From the stretch amplitude and the force response,
stiffness was calculated as the ratio of the change in force to the change in length.

Immediately following the stiffness recording, isotonic force during isokinetic shortening at 2 muscle lengths per second was determined ($F_{\text{isk}}$) (Fig. 1). This velocity was selected as it is close to that at which frog muscle produces maximal power at room temperature (personal observation). During the plateau of an isometric contraction, the muscle was quickly shortened by about 1% muscle length; this caused force to drop close to the level at which it would stabilize during subsequent isokinetic shortening. After the shortening step the muscle was then ramp shortened at 2 muscle lengths per second, and isotonic force was recorded. Stimulation continued throughout the step and isokinetic shortening. The step shortening amplitude determined to be appropriate to measure $F_{\text{isk}}$ during pre-fatigue (control) conditions was also used during fatigue and recovery. There was not time during the fatigue/recovery recordings to continually re-establish the ideal step size, however it remained adequate throughout the experiment.

The work loop technique was used to measure cyclic work output as may occur during cyclic movement in animals (26). Muscle length was cycled in a sinusoidal fashion while imposing phasic stimulation. The length cycle frequency was set to 4 Hz and the amplitude of the length cycle was set to 15% of muscle length (peak-to-peak). These conditions yield close to maximal power at room temperature in frog muscle (unpublished observation). The stimulus duration and phase required to maximize net work output were determined at the beginning of each experiment, and were always close to 100 ms and 58° respectively (where 90° is the point in the sine cycle where the muscle is at maximum length, and 270° is the point of minimum length). The muscle’s length was cycled and it was so stimulated for 3 consecutive cycles, and work was measured from the second or third cycle in the set. When the muscle fatigued it became
progressively stiffer with each cycle in the set of 3 and so force and work per cycle failed to stabilize; in these cases work was taken from the third cycle which was the closest to a steady state condition. Three different measures of cyclic work were assessed: i) shortening work is the integral of muscle force with respect to muscle length over the shortening portion of the cycle (i.e. the work done by the muscle while it shortened), ii) lengthening work is the integral taken over the lengthening portion of the cycle (i.e. the work required to stretch the muscle), and iii) net work is the integral over a complete lengthen/shorten cycle (i.e. the net work done by or absorbed by the muscle during a complete cycle) and is the difference between shortening work and lengthening work. A positive value for net work indicates the muscle is imparting energy to the system, while a negative value indicates the muscle is absorbing more work than it contributes.

fatigue and recovery

After all parameters had been established (see previous section), a set of control recordings of Po, twitch force, stiffness, F_{isk}, and cyclic work were made. These measurements were taken several times over a period of about 20 minutes to ensure the preparation was stable. The muscle was then given isometric, tetanic stimulation (100 Hz for 150 ms duration) repeatedly with 2 or 5 second intervals to induce fatigue; most muscles did not show appreciable fatigue unless stimulated with a 2 second interval. Such stimulation was continued until Po declined to 50% of its control value. Po was recorded on the computer each time it declined by approximately 2% of its initial level. Recordings of cyclic work, stiffness, F_{isk}, and twitches were made each time Po declined by about 5%. Measurements of either cyclic work or the combination of (F_{isk} + stiffness + twitch) were alternated to prevent excessive recovery or fatigue
of the muscle between records of Po. Thus, a typical sequence would include several measures of Po, then cyclic work, then several more of Po, then \((F_{isk} + \text{stiffness} + \text{twitch})\), then more of Po, then cyclic work, etc. Once Po had fatigued to 50% of control, the muscle was allowed to recover. During recovery the muscle was stimulated less frequently; every 30 s initially and up to 5 minutes apart during the latter stages of recovery. Again, measurements of Po were made in all cases, and paired with alternate measurements of cyclic work or \((F_{isk} + \text{stiffness} + \text{twitch})\). After the muscles had recovered, they were removed from the chamber, trimmed of any tendon and loose tissue, blotted on filter paper and weighed.

\textit{data analysis}

All of the measurements for each muscle were initially plotted as a time series. The relative changes with fatigue and recovery were found to be strikingly consistent between experiments for all but a few of the measurements (see below). The data sets were thus combined for analysis using the following procedures. To control for differences in the sizes of the preparations, all data were first standardized as a percent of the control (i.e. pre-fatigue) values, where control is 100%; note that some measurements actually exceed 100% of control by a small amount due to potentiation during the very early stages of fatiguing stimulation. Despite standardizing the data so, it was not possible to then simply average measurements across preparations because not all measurements were made at the same time after the onset of stimulation nor at precisely the same level of fatigue. Further, such a presentation is biased by the specific stimulation protocol chosen to fatigue the muscle, complicating interpretation. We therefore expressed all the data relative to the level of Po during fatigue and recovery as described next.
In all experiments Po started at 100% and declined to 50% before recovering again, thus the data is expressed as a function of declining Po during fatigue and increasing Po during recovery. Recordings of Po were interspersed with recordings of Twitches, cyclic work, F_{isk}, and stiffness. As all the measurements could not be made simultaneously, we estimated their values at each moment that Po was recorded using linear interpolation as follows. Associated with every measure of Po, a value for each of the other measures was calculated using recorded values that bracketed the measure of Po and assuming a linear change with time between them. For example, if the recording of Po occurred at a time exactly half way between two recorded values of data, the value of the predicted data point (associated with the time that Po was recorded) would be exactly half way between the two bracketing values. The result was a series of values for Po recorded during fatigue and recovery, and associated values for each of the other measurement as predicted to have occurred if measured at the same moment as Po. The data from all experiments were then combined and binned based on 5% changes of Po during fatigue and during recovery. Data were not normally distributed and were compared using a Kruskal-Wallis one-way ANOVA on ranks and Dunn’s multiple comparisons. Differences from control were considered significant with p<0.05. All data are presented as mean ±SEM.

**Stiffness and corrected stiffness**

The proportion of the cross-bridge population in the attached state during contraction can be estimated from muscle stiffness. This requires knowledge of the stiffness of the muscle during rigor, where it is assumed that 100% of the available, cycling cross-bridges are attached, and knowledge of the proportion of the recorded stiffness that originates in the cross-bridges themselves. It was assumed (i) that the stiffness of unfatigued frog muscle in rigor is 1.63 fold
greater than during maximal tetanus (29), (ii) that 50% of the total compliance resides in the
cross-bridges, which agrees well with an estimate of 43% (29), (iii) that each myosin head
contributes the same increment to stiffness in active contraction as it does in rigor (40) and (iv)
that this stiffness is independent of the state of fatigue. The proportion of attached cross-bridges
during isometric tetanus was then estimated as \( \frac{S_A}{S_R} / (2 - \frac{S_A}{S_R}) \), where \( S_A \) is the stiffness
during active, tetanic contraction (as measured during fatigue and recovery) and \( S_R \) is the
stiffness in rigor.

These assumptions are not entirely valid. The origin and distribution of compliance in
muscle is not entirely certain, thus stiffness may not be a quantitatively reliable predictor of the
absolute proportion of attached cross-bridges (21). However, these are not likely to change
appreciably with fatigue, and thus changes in corrected stiffness should be indicative of changes
in the population of attached cross-bridges. A greater hurdle to interpretation is that the stiffness
and force generating capacity of individual cross-bridges are sensitive to their nucleotide state,
they may be differentially so, and that the stiffness of a cross-bridge in rigor may depend on how
the rigor state is achieved (e.g. 8,34,38). For example, force falls more rapidly than stiffness as
the level of phosphate rises, thus the relationship between force and stiffness may not be linear
during fatigue and would violate an assumption above. Values and relative changes in
proportions of attached cross-bridges so estimated should thus be interpreted with caution; as
such we refer to our estimate as ‘corrected stiffness’, but imply that it approximates the
proportion of attached cross-bridges.
RESULTS

Data are presented for muscles from 7 animals. Po was 216 ±21 kN m⁻² before the fatigue protocol. Young’s modulus at maximum force was 5.08 ±0.48 MPa before fatigue. Net, cyclic work output was 15.5 ±4.25 Jkg⁻¹ and power was 61.9 ±17.0 Wkg⁻¹ at a cycle frequency of 4 Hz and 15% strain, typical for frog muscle at room temperature (44,45).

With the fatigue protocol used, Po declined by 50% and then recovered toward initial levels over an approximately 30 minute period; fatigue lasted about 10 minutes and recovery about 20 minutes. There was no obvious decline in the rate of fatigue of Po at the height of fatigue, while the rate of recovery slowed as recovery progressed. Muscles recovered to an average of 88.3 ±1.6% of control Po after 20 minutes, with the poorest recovery being 83%. This level of recovery is similar to that reported by Thompson et al. (50) and Fitts & Holloszy (20) in frog sartorius and semitendinosus after 30-40 minutes of recovery from a 50-90% reduction in Po. No attempt was made to leave the muscles in the chamber for prolonged periods to determine if full recovery would eventually occur as the gradual and unavoidable deterioration of isolated preparations would render such evidence inconclusive. Thus, while most of the decline in force was due to reversible fatigue, we can not rule out a small contribution from cell death or damage.

Two exemplary data sets are plotted versus time during fatigue and recovery to assess patterns (Fig. 2). Po, stiffness, Fisk, and shortening and net work all declined during fatigue and then recovered with trajectories that were similar across preparations. Lengthening work and measures of the twitch duration increased during fatigue and then decreased back toward initial levels during recovery. However, two distinct patterns for recovery of twitch relaxation were noted in different preparations. In 3 of 7 preparations, the period of twitch relaxation declined rapidly during early recovery and then continued to decline more gradually as recovery
continued (Fig. 2D). The other 4 preparations showed a similar, early, rapid recovery in the period of twitch relaxation, but then a substantial rebound in duration as recovery continued, reaching values similar to those at the height of fatigue (Fig 2B). Twitch relaxation eventually returned toward control levels during the later stages of recovery in all preparations. The transient increase in twitch duration during recovery noted in some preparations resulted from the development of a pronounced secondary slowing of relaxation about mid-way through the relaxation phase, and thus does not reflect a simple reversal of the recovery process.

Twitch force initially increased and then decreased back toward control levels during fatigue (Figs. 2A,C). During recovery there was a transient increase in twitch force, the magnitude of which varied between preparations from virtually none (Fig. 2A) to levels that rivaled the maximum attained during fatigue (Fig. 2C). Of note, in muscles that showed the recovery/rebound/recovery of both twitch force and the period of twitch relaxation, the transients were not synchronized with one another. Also, in some cases one measure but not the other showed the pattern of rebound during recovery.

In the remaining graphs data are expressed relative to Po as described in the methods. Two lines are shown on each graph, one showing data during fatigue (downward, filled triangles with solid lines) and one during recovery (upward, open triangles with broken lines). The line of unity is a thin, dotted line; data falling above this line indicate the relative change in a particular measurement was less than the decrease in Po, and data falling below the line indicate the change was more than the decrease in Po.

Results for the two steady-state measures, stiffness and Fisk, are described first (Fig. 3). As Po fatigued toward 80% of control, corrected stiffness remained unchanged. With further fatigue of Po to 50%, corrected stiffness decreased in an approximately linear fashion to about
75% of initial levels, eventually changing to become parallel to the line of unity with Po. Corrected stiffness recovered with a trajectory that lagged slightly that during fatigue. $F_{isk}$, the force produced during isokinetic shortening, reflects the muscle’s ability to do work under conditions of maximal, sustained activation. $F_{isk}$ declined linearly with fatigue of Po, but declined to about 20% of its control level despite Po dropping only 50%. $F_{isk}$ recovered with a trajectory that slightly lagged that during fatigue.

The work done by the muscle during cyclic activity, as might occur during locomotion or other repetitive movements, was measured using the work-loop technique. Before and during early fatigue, the force produced by the muscle during shortening (while the muscle was stimulated) was greater than the force required to lengthen it (while it was not stimulated), resulting in a work-loop that was traversed in a counter-clockwise direction and net work production by the muscle during a cycle (Fig. 4 inset). As fatigue ensued, the force produced by the muscle during shortening decreased and the force required to lengthen the muscle increased, the latter due to relaxation after activation becoming progressively less complete (Fig. 4 inset); the force during lengthening eventually exceeded the force during shortening so that the work-loops were traversed in a clock-wise direction and the muscle absorbed net energy during each cycle (i.e. more work was required to lengthen the muscle than was done by the muscle when it shortened). Of note, as a result of incomplete relaxation in fatigued muscle the force during the early stages of muscle shortening (i.e. immediately after stretch) exceeded the force at this point in unfatigued muscle (Fig. 4 inset). This resulted in more work being done by the fatigued muscle than unfatigued muscle during the initial stages of shortening. As such, while shortening work during cyclic activity did decrease with fatigue, to about 70% of control as Po declined to 50%, it rose further above the line of unity with Po as fatigue progressed (Fig. 4), reflecting the
progressively incomplete relaxation and increased force at the onset of shortening.

Also as a result of this progressively incomplete relaxation, the work required to lengthen the muscle increased rapidly during the early stages of fatigue, reaching 600% of control when Po had declined by only 20% (Fig. 5). Lengthening work peaked at about 800% of control as Po continued to decline toward 50%. During the initial recovery of Po (from 50 to about 70%), lengthening work remained steady at about 500% of control, after which there was a rapid and almost complete recovery of lengthening work as Po recovered from 70 to only about 80% of control. Interestingly, the entire fatigue of lengthening work occurred as Po declined from 100 to 80% of control, while almost the entire recovery of lengthening work occurred as Po recovered from 70 to 80% of control. Thus, the major effects occurred at relatively low levels of fatigue and over a very small range of Po. The net cyclic work output, which is the net mechanical work produced by the muscle during a complete lengthen/shorten cycle, fatigued very rapidly, reflecting the steady decline in shortening work and the rapid rise in lengthening work. Net work declined to zero when Po had fatigued by only 15-20% (Fig. 6). Net work continued to decline with further fatigue, becoming negative and then reaching a minimum as Po fatigued to only about 70% of control. Net work recovered somewhat more rapidly than it fatigued relative to Po; when Po had recovered to 80% of control net work had recovered to about 50%, despite being zero at the same level of Po during fatigue. Net work was always below the line of unity with Po during fatigue and recovery.

Isometric twitch force was well above the line of unity with Po at all times during fatigue and recovery (Fig. 7). Twitch force showed an initial increase of about 50% with fatigue, and then gradually declined back towards control levels with further fatigue. The extent of the increase of twitch force and the level to which it returned at the height of fatigue were quite
variable; in some preparations twitch force was still substantially above its control level at the height of fatigue. During recovery, twitch force also showed a transient increase in all preparations, the magnitude of which varied from very little to levels that equaled the same high forces achieved during early/mid fatigue (Figs. 2A,C). However, because twitch force fell below control levels during recovery in some preparations, when averaged across all preparations the transient rise was not readily apparent nor statistically different from control (Fig. 7). While difficult to discern in the averaged plots, the transient rise of twitch force during both fatigue and recovery occurred over the same range of values of Po.

The duration of the twitch during force development, measured as the time from the stimulus to peak force, increased in an approximately linear fashion to about 180% of control as Po declined by 50% (Fig. 8A). It recovered along a remarkably similar trajectory. An alternate measure of the duration of the twitch during force development is the time from 10 to 90% peak force, which displayed the same pattern during fatigue as did the period from stimulus to peak force just described (Fig. 8B). The duration of the twitch during relaxation, measured as the time from 90 to 10% of peak force, also increased nearly linearly with fatigue of Po, but to over 400% of control (Fig. 8C). Like the previous measures, the duration of relaxation recovered along a trajectory very similar to that during fatigue, with the notable exception of some preparations in which there was a large, transient increase during the late stages of recovery (as noted above).

**DISCUSSION**

While we do not address directly the cellular mechanisms of fatigue in this study, we present here a brief summary of proposed mechanisms which may be supported or refuted by, and are relevant to understanding, changes in mechanical performance with fatigue. The changes
that occur during fatigue are held to follow a series of stages, each reflecting its own characteristic failure of some element in the chain of excitation through cross-bridge activity. The initial fall in force during fatigue from high frequency stimulation, from rest (100%) towards about 75%, is attributed to an impairment of the ability of cross-bridges to generate force, with little or no change in the free calcium transient (activation) and by crude inference the number of attached cross-bridges (17,19,54). This initial impairment of cross-bridge force has been attributed to increased proton loads and accumulation of inorganic phosphate (9,10,19,23,36,54,55,58). Acidification of frog muscle reduces force and unloaded velocity of shortening as well as slowing twitch kinetics (18,43), but does not explain entirely the effects of fatigue (32). The effects of acidification appear to be quite limited in mammalian muscle at normal temperatures (e.g. 37,42,57), making inorganic phosphate a prime candidate as the inhibitor of not only force and shortening velocity during fatigue, but also intracellular calcium handling and myofibrillar calcium sensitivity (1,10,11,27,54,55). High energy phosphate depletion during fatigue does not appear to be a factor that limits cross-bridge function (12,19,35). If activity continues past the early stages of fatigue, the muscle suffers a subsequent fall in force that is attributed to reduced calcium release from the sarcoplasmic reticulum and thus reduced activation and fewer cross-bridges, again perhaps due to accumulation of Pi (2,14,27,53,54,56,58) and failure of the inward spread of activation (17,22). Sustained activity may also be associated with physical damage at the triads and ryanodine release channels, leading to impairment of calcium handling that recovers with a time course of hours or days (56).
Effects on Tetanic Force

During the early, rapid fall of force with fatiguing stimulation (or the initial linear fall of force in cane toad muscle) the internal free calcium transient remains normal or may actually increase (27,51,52,54). Therefore, excepting a potential effect of fatigue on troponin affinity for calcium (reviewed in 19), the level of activation and hence number of cross-bridges should not be greatly impaired in early fatigue. Our stiffness results (Fig. 3) and those of others (9,16,17) support this notion. Thus, after moderate fatigue there is only a small reduction in the number of cross-bridges, and the fall in force is attributed largely to a reduction in the average force per attached cross-bridge. Here we also report stiffness during the very initial stages of fatigue (from rest to about 80% Po) and found that it remained unchanged (Fig. 3). Hence, the initial fatigue of Po appears to be due entirely to less force per cross-bridge. A reduced ability of cross-bridges to generate force has been attributed to accumulation of phosphate (19,36,55) and more so to protons in frog muscle (e.g. 18,19,23,39).

Fatigue below 80% Po was accompanied by a steady decline in stiffness (Fig. 3) (17), with the rate of decline eventually matching the rate of fatigue of force. This supports suggestions that during more severe fatigue there is failure of activation and thus impaired recruitment of cross-bridges via accumulation of protons and phosphate associated with inhibition of calcium release and reuptake by the sarcoplasmic reticulum (2,14,19,27,51,54,55,56), impairment of the inward spread of excitation (17,22), and failure of excitation contraction coupling at the triads (6).

The ratio (Po / corrected stiffness) is an index of the average force produced per cross-bridge. During fatigue, the force per cross-bridge declined in an exponential fashion (Fig. 9), being near the line of unity with Po during early fatigue (control to 80%), but approaching a
predicted minimum of 69% of control with further fatigue (see Fig. 9). In combination with corrected stiffness (Fig. 3) these data suggest i) an immediate impairment of the ability of cross-bridges to generate force as fatigue commences, accounting entirely for the decline in force during early fatigue, ii) an eventual lower limit to the force per cross-bridge with advanced fatigue (about 69% of control this study), and iii) the decline in Po during moderate and severe fatigue is attributed increasingly and eventually entirely to a decline in the number of attached cross-bridges (i.e. impaired activation). Again, potential non-linearities between stiffness and force or proportion of attached cross-bridges as a result of fatigue leave these conclusions tentative, less so during early and more so during later fatigue.

During recovery, force per cross-bridge increased more rapidly while the number of cross-bridges increased more slowly than during fatigue (Figs. 3 & 9). Further, the force per cross-bridge fatigued exponentially but recovered linearly with respect to Po. Further still, at levels of Po between 80 and 100% of control, stiffness remained unaltered during fatigue but was depressed during recovery. These differences strongly suggest that the mechanisms responsible for altering the number of attached cross-bridges versus the force per cross-bridge are independent (such as accumulation of protons or phosphate versus impaired activation), that they fail and recover at different rates, and that Po itself cannot be used directly to infer the cause of fatigue.

**Effects on Twitches**

We use the term activation to refer to most aspects of excitation contraction coupling, including the time course and magnitude of the calcium transient and the associated activation of thin filaments. While this use is imprecise, we intend it only as an ellipsis of the processes
involved. The amplitude of the intracellular free calcium transient increases during the early stages of fatigue (saturation of myoplasmic calcium buffers), and then gradually declines as fatigue progresses (reduced calcium release from the sarcoplasmic reticulum) (1,19,51). The duration of the free calcium transient also becomes prolonged during fatigue (52,59) and the resting intracellular calcium levels rise, causing a prolongation of the duration of the twitch, particularly the relaxation phase (1,51,59). See MacIntosh et al. (30) for a discussion of the effects of fatigue on twitch duration and its interpretation. Twitch force and kinetics should reflect these changes in activation (50), which in turn should influence the ability of muscle to power locomotion.

While there is commonly a decline in twitch force during fatigue (e.g. 20,50), a transient rise during early fatigue is not novel (20). In the present study, twitch force increased during fatigue and reached a maximum about 50% above control (Figs. 2 & 7), this in the face of a 20% decline in force produced per cross-bridge (Fig. 9) and no change in corrected stiffness during tetanus (Fig. 3). Other factors affecting peak twitch force include the magnitude and time course of activation, cross-bridge kinetics and the series compliance. We assumed the series compliance to be passive and not prone to fatigue, however it is possible that in a muscle or fiber bundle its distribution and thus characteristics could change during fatigue. Regarding cross-bridges, slowing of the cross-bridge detachment rate with fatigue as reflected in a slowing of the maximal velocity of shortening (both Vmax and Vo) (9,18) would cause an increase in the number of attached cross-bridges and hence twitch force. However, there is also a slowing of twitch dP/dt (20,50) suggesting a slowing of the attachment rate which would result in less force (see also 19), and there was no change in stiffness during early fatigue (Fig. 3) suggesting no change in the number of cross-bridges. As cross-bridge kinetics during the plateau of a tetanic contraction
are different than during the early portion of a twitch, conclusions about twitches based on results from tetanic contractions (stiffness, Vmax) should be interpreted with due caution. Lastly, an increase in amplitude of the twitch free calcium transient during early fatigue (see above) could result in more force. Likewise, prolongation of the calcium transient would lead to a longer twitch (50), allowing more time for cross-bridges to form and extend the series compliance (as suggested by Metzger & Fitts, 33). In support of this idea, Sun et al. (48) noted a close relationship between the rate of decay of the calcium transient and peak twitch force. Similarly, during the early stages of fatigue (100 to 80% Po) we noted a highly significant, linear relationship (slope = 0.90, P<0.001) between peak twitch force and twitch duration (measured as the time available for force development, 10-90% peak force); however a low r² for this relationship (0.39) suggests other factors are also involved. Thus, the increased twitch force during early fatigue seems attributable to the increased time available to develop force.

As fatigue progressed further, twitch force tended back toward control levels (Fig. 7). Associated with this drop in twitch force was a 30% decline in corrected stiffness (fewer cross-bridges) (Fig. 3) and a 10% decline in force per cross-bridge (Fig. 9); together these approximately account for the drop in twitch force. On average, twitch force remained remarkably stable near control levels during the entire period of recovery (Fig. 7). Thus the near linear increase in corrected stiffness (Fig 3) and force per cross-bridge (Fig. 9) during recovery, which would increase twitch force, appeared to counteract the effects of a near linear decline in twitch duration (Fig. 8), which would decrease twitch force. Of note, the large separation between fatigue and recovery of twitch force when expressed relative to Po (Fig. 7) is strong evidence that different mechanisms are responsible for the impairment of tetanic force versus twitch force.
The present results suggest that during early fatigue the continued slowing of calcium kinetics augments twitch force and this overshadows the reduced force per cross-bridge, while later in fatigue the combination of reduced force and number of cross-bridges begins to dominate. During recovery, cross-bridge force and number and activation kinetics interact such that force remains relatively stable. Given that fatigue of twitch force involves changes in the force per cross-bridge, number of cross-bridges, the kinetics and level of activation, as well as the effects of potentiation, it may not be surprising to find extreme variability in the response of twitch force to fatiguing stimulation, both between preparations and fatigue protocols.

Slowing of the twitch during fatigue is commonly observed (see also 11), and is attributed to a slowing of the calcium transient (reviewed in 19). The periods of both twitch contraction and relaxation showed an increase during fatigue and decrease during recovery, were near linearly related to Po at all levels of fatigue, and there was a striking similarity during fatigue and recovery to their relationship with Po (Fig. 8). This linear and consistent relationship between twitch duration and Po has not been reported previously, and implies that either a single element acting via different mechanisms, or a single mechanism itself is responsible for changes in both Po and twitch duration. While there is evidence that failure of activation results in reduced tetanic force during severe fatigue, it does not appear to be an important factor during early fatigue (14) and is thus unlikely to account for the similar changes in both twitches and Po at all stages of fatigue. Both twitch kinetics and Po are influenced by the interactions of calcium with troponin and by cross-bridge kinetics (41), however we are not aware of compelling evidence that either could explain the correlated changes observed. Alternatively, proton or inorganic phosphate accumulation may be single elements that could similarly influence both twitches and Po via different mechanisms; they simultaneously reduce force and slow
intracellular calcium kinetics (5,55, reviewed in 19), although a clear affect on the calcium transient is not yet demonstrated. Changes in Po are a complex function of changes in both the number of and force generated by cross-bridges, which we have noted above appear to involve separate mechanisms; likewise twitch duration is a complex function of compliance and the kinetics of activation and troponin affinity, which are all independent. Thus, whether a sole agent or many mechanisms acting in concert are responsible, the linear tracking of twitch duration and Po during fatigue and recovery seems extraordinary.

Effects on Work

When fatigue is assessed in the context of cyclic animal movement, changes in twitch duration and mechanical work or power are of primary concern. Prolongation of the twitch will severely hinder movements that require rapid oscillations in force, even in the face of a maintained ability to produce force (41,49). Stevens & Syme (46) first highlighted the marked difference between fatigue of Po and the ability to perform cyclic work, and conclude that Po is a poor index of a muscle’s ability to do work in a system undergoing cycles of flexion and extension. In accord, we note here that net work output declined precipitously to zero as Po declined by only 20%, and continued to decline to -50% of control (net work absorption) when Po dropped by less than half (Fig. 6). Thus, although the muscle was capable of producing considerable amounts of tetanic force at the height of fatigue, more work was required to lengthen the muscle than was done during shortening. This would be disastrous for certain movements, particularly during undulatory swimming, where the muscles used for propulsion are also the antagonists of contralateral muscles. If muscles on both sides of the animal fatigue at the same rate, as may be expected, the animal would become paralyzed when net work
reached zero, despite their muscles retaining most of their ability to produce force. While the experimental use of isolated preparations precludes selective and graded recruitment of motor units as employed by animals, it is unlikely that the tendency for net work to become negative with fatigue could be avoided without a drastic curtailment of power output. Regardless of the strategy used to activate the muscle, rapid failure of the system to power locomotion will ensue.

Even animals with dedicated antagonistic muscles, where one muscle powers movement and is extended by an antagonist that does not contribute directly to movement, likely do not fare a great deal better. In such cases it is only the work done by the power producer while shortening that is relevant to propulsion; the work to extended them is done by their antagonist and does not detract from propulsion. Yet the work to extend the power producer increased to near 600% of control as Po fatigued by only 20% (Fig. 5). The power producer's antagonist will then fatigue against this high load and suffer the same escalation in work required to extend it; work that comes at the expense of its antagonist, which is the muscle powering movement itself. Hence, it is again net work that is relevant; the energy available for propulsion is the difference between the shortening work done by the muscle powering movement and the work required to extend its antagonist. Thus, the power available for propulsion fatigues much more rapidly than either the ability to produce force or shortening work. Further, the energetic cost to sustain movement during fatigue will be extreme, including the cost of powering movement and the rising costs of extending both the propulsive muscle and its antagonist. Paradoxically, the height of energy consumption could occur under conditions where mechanical power available for movement is minimal; antagonistic muscles would expend most of their energy simply extending one another, with little or nothing left to power locomotion.
Quite unlike net work, cyclic shortening work actually failed less than Po as fatigue progressed (Fig. 4)(see also 46). This reflects the progressive lack of complete relaxation of the muscle during fatigue (Fig. 4 inset)(see also 46). Incomplete relaxation led to much higher forces during the stretch portion of a work cycle, thus higher forces and more work done at the subsequent onset of shortening; more than might be expected based on Po. This failure of relaxation and enhancement of cyclic shortening work also caused a notable difference between the effects of fatigue on the dynamic measure cyclic shortening work, and the steady-state measure of shortening work $F_{isk}$. Work during isokinetic shortening (proportional to $F_{isk}$) (Fig. 3) declined much more than shortening work during cyclic contractions (Fig. 4), to the extent that isotonic work was only 30-50% of cyclic work at the height of fatigue (Fig. 10). This was initially unexpected, as unlike $F_{isk}$, cyclic work is additionally limited by activation kinetics (7); we anticipated that these limitations would be exacerbated as fatigue progressed. However, as noted above, cyclic shortening work was actually enhanced by the failure of the muscle to relax fully when fatigued, while $F_{isk}$ is independent of activation kinetics and was not. The enhancement was so pronounced as to bias cyclic work upwards by 2-3 fold over what was predicted based on steady-state measurements using $F_{isk}$. This again reinforces the important difference between cyclic and steady-state measures of performance, even when both measures are of the work done when a muscle shortens.

The rate of doing work is power, which is the product of force and shortening velocity. A shift in the force-velocity relationship of muscle after fatigue has been reported (e.g. 25,58) such that when maximum power from force-velocity curves is expressed relative to the product of $V_{max}$ and Po there is a 10-50% increase in relative power output with fatigue (i.e. the decrease in the ability to produce power with fatigue is less than the decrease in the product of Po and
Vmax) (3,9,13). The decline in Po with fatigue has been noted to be similar to, somewhat less than or greater than the decline in Vmax (3,9,18,20,25,50,58). F_{isk} in the present study is one point on the force-velocity relationship and reflects power during isokinetic shortening with maximal activation. While we did not specifically select F_{isk} to produce maximal power, we did select it to be close (see methods); accepting this, and based on the observation that at the height of fatigue Po declined to 50% and F_{isk} (power) to 25%, then Vmax would also have to decline by at least 50% for relative power to increase, similar to reports above. The approximately equivalent drop in ability to produce force and shorten with fatigue, and the linear and virtually identical relationship between Po and F_{isk} during both fatigue and recovery (Fig. 3) suggest a common mechanism may be responsible for changes in force production and the ability to shorten during fatigue.

In summary, early fatigue was attributed entirely to a reduction in the ability of cross-bridges to generate force, and was followed and accompanied by a reduction in the number of cross-bridges with further fatigue. The force per cross-bridge reached a lower limit at about 69% of maximum, whereby further reductions in force with fatigue were attributed entirely to a loss in the number of cross-bridges. Fatigue was accompanied by a slowing of contraction kinetics, which appears to be the primary factor responsible for differences between dynamic versus steady-state measures of performance during fatigue. Because slowed relaxation has no influence on steady-state performance such as Po or F_{isk}, changes in these measures with fatigue reflect only changes in cross-bridge function. They insinuate the effects of fatigue on animal movement, but in an indirect and often misleading fashion. Slowed relaxation was a major contributor to all aspects of changes in dynamic performance. The drastically reduced net work output and increased lengthening work during cyclic contractions, the augmentation of work done while
shortening during cyclic contractions above what would be predicted based on Po or Fisk, the longer twitch duration and the increased twitch force despite fewer and weaker cross-bridges all have a basis in slowed activation kinetics and are all relevant to animal movement. That failure of Po by a mere 10% would signify that antagonistic pairs of muscles have been rendered almost incapable of producing any net power, or that in spite of this the same muscles are actually doing more work when they shorten than Po or Fisk would predict, is evidence that dynamic measures of performance are uniquely important to understanding the effects of fatigue on animal movement. Steady-state measures of muscle performance, which do not reflect activation kinetics and dynamic properties of contraction, will not provide accurate information about the impact of fatigue on a muscle's ability to power movement.

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REFERENCES


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Figure Legends

Figure 1. Measurement of isometric force (Po), stiffness and isotonic force during isokinetic shortening ($F_{isk}$).

Muscle was held isometric and stimulated tetanically at 100 Hz until force reached a plateau. A step stretch of 0.3% muscle length was then applied (upward arrows); the ratio of change in force to change in length is the uncorrected stiffness. The muscle was then shortened back to the original length, held until force stabilized, and then isotonic force during isokinetic shortening at 2 muscle lengths per second was measured ($F_{isk}$) (downward arrows).

Figure 2. Exemplary plots of measurements made during fatigue and during recovery.

The period of fatiguing stimulation is indicated by the black bar (see methods for details). Left panels (A and B) and right panels (C and D) show results from two different muscles. Work required to lengthen the muscle (upward triangles and broken lines) is referred to the right axis of the upper panels; all other measures (symbols and solid lines in legend) refer to the left axes. Of note, the muscle in panels A and B showed only a small rebound of isometric twitch force during recovery, but a very large transient increase in the period of twitch relaxation (90-10% of peak force), while the muscle in panels C and D showed a very large rebound of twitch force during recovery but virtually no transient increase in the period of twitch relaxation.

Figure 3. Steady-state measures of muscle performance during fatigue and recovery.

Corrected stiffness (upper panel) represents a relative proportion of attached cross-bridges (see methods). Isokinetic force ($F_{isk}$) while shortening at 2 muscle lengths per second (lower panel) was measured after the plateau of isometric, tetanic contractions had been attained, and is
equivalent to work during shortening. Values are expressed relative to the control (prefatigue) condition. Solid lines and filled downward triangles are during fatigue; broken lines and open upward triangles are during recovery. The line of unity is a dotted line. Data are mean ± SEM. Asterisks indicate values that are significantly different from control (P<0.05).

**Figure 4. Shortening work done during cyclic contractions versus isometric, tetanic force during fatigue and recovery.**

Work was measured using the work loop method; cycle frequency was 4 Hz and strain was 15% of muscle length. Values are expressed relative to the control (prefatigue) condition. Solid lines and filled downward triangles are during fatigue; broken lines and open upward triangles are during recovery. The line of unity is a dotted line. Data are mean ± SEM. Asterisks indicate values that are significantly different from control (P<0.05). Inset shows exemplary work loops before fatigue (solid line), mid-way through fatigue (broken line) and at the height of fatigue (dotted line). Arrows indicate the direction the loops are traversed; note the control loop runs counterclockwise while the fatigued loops run clockwise.

**Figure 5. Work required to lengthen the muscle (absorbed) during cyclic contractions versus isometric, tetanic force during fatigue and recovery.**

Work was measured using the work loop method; cycle frequency was 4 Hz and strain was 15% of muscle length. Values are expressed relative to the control (prefatigue) condition. Solid lines and filled downward triangles are during fatigue; broken lines and open upward triangles are during recovery. The line of unity is a dotted line. Data are mean ± SEM. Asterisks indicate values that are significantly different from control (P<0.05).
Figure 6. Net work done by the muscle during cyclic contractions versus isometric, tetanic force during fatigue and recovery.

Net work was measured using the work loop method and is the area enclosed by the loop formed when plotting force versus muscle length during a complete cycle (see fig 4 inset); cycle frequency was 4 Hz and strain was 15% of muscle length. Positive values indicate the muscle did more work during shortening than was required to lengthen it, while negative values indicate more work was required to lengthen the muscle than it did during shortening. Values are expressed relative to the control (prefatigue) condition. Solid lines and filled downward triangles are during fatigue; broken lines and open upward triangles are during recovery. The line of unity is a dotted line. Data are mean \( \pm \) SEM. Asterisks indicate values that are significantly different from control (P<0.05).

Figure 7. Isometric twitch force versus isometric, tetanic force during fatigue and recovery.

Twitch force is the difference between peak and resting force. Values are expressed relative to the control (prefatigue) condition. Solid lines and filled downward triangles are during fatigue; broken lines and open upward triangles are during recovery. The line of unity is a dotted line. Data are mean \( \pm \) SEM. Asterisks indicate values that are significantly different from control (P<0.05). Inset shows exemplary twitches before fatigue (solid line), mid-way through fatigue (broken line) and at the height of fatigue (dotted line).
Figure 8. Three measures of twitch kinetics versus isometric, tetanic force during fatigue and recovery.

Panel A shows the time from the stimulus to peak twitch force. Panel B shows the time from 10% to 90% of maximum twitch force during contraction. Panel C shows the time from 90% to 10% of maximum twitch force during relaxation. Values are expressed relative to the control (prefatigue) condition. Solid lines and filled downward triangles are during fatigue; broken lines and open upward triangles are during recovery. The line of unity is a dotted line. Data are mean ±SEM. Asterisks indicate values that are significantly different from control (P<0.05). The two outliers are preparations that showed unusually high twitch durations late in recovery.

Figure 9. Force per cross-bridge versus isometric, tetanic force during fatigue and recovery.

Force per cross-bridge was estimated as the ratio of tetanic force to corrected stiffness (see methods). Values are expressed relative to the control (prefatigue) condition. Solid lines and filled downward triangles are during fatigue; broken lines and open upward triangles are during recovery. The line of unity is a dotted line. The interrupted line is a single order exponential curve fit to the data during fatigue (0.29Ae^{0.046P0} + 68.4). Data are mean ±SEM.

Figure 10. Work done while shortening during cyclic contractions versus isokinetic force (F_{isk}) during shortening at 2 muscle lengths per second during fatigue and recovery.

When ramp shortening, F_{isk} is proportional to work done and reflects the ability of the muscle to do work during sustained, maximal activation. Cyclic shortening work reflects the ability of the muscle to do work during cycles of cyclic activation and length change. Values are expressed
relative to the control (prefatigue) condition. Solid lines and filled downward triangles are during 
fatigue; broken lines and open upward triangles are during recovery. The line of unity is a dotted 
line. Data are mean \( \pm \) SEM.
Fatigue
Recovery

Tetanic Force (% control)

Corrected Stiffness (% control)

Isokinetic Force (% control)

Cyclic Shortening Work (% control)

Length (mm)

Force (mN)

Fig. 3

Fig. 4
Fig. 5

Cyclic Lengthening Work (% control)

Tetanic Force (% control)

Fig. 6

Cyclic Net Work (% control)

Tetanic Force (% control)