Introduction

Over the last decade exercise has been promoted as an important contributor to a healthy lifestyle. However, due to various complications (e.g. heart disease or obesity) certain populations of people are unable to exercise. One of the chief complaints for their inability or reluctance to exercise is fatigue. Skeletal muscle fatigue can be defined as the inability to maintain a desired power output during repeated contractions (9). Fatigue also poses a significant clinical problem in that it increases susceptibility to orthopedic and musculotendinous injury and often leads to the development of muscle soreness (38). It is possible that the development of fatigue can prevent people from accomplishing everyday activities which may lead to a reduction in their quality of life. There can be many influences upon one’s inability to sustain physical activity, such as environment (e.g. altitude or temperature), disease state (e.g. elevated blood pressure) and diet (e.g. carbohydrates or water intake) (30). With all of the potential variables it is difficult to attribute just one single cause for the inability of a person to sustain a desired muscular power output to meet a particular activity requirement.

Muscle fatigue has been studied for many years and many ideas have been proposed to be a cause or contributing factor to the fatigue of skeletal muscle. As early as 1847, J. Berzelius recognized that amount of free lactate in muscle was proportional to the extent in which the muscle has been exercised. This led to Fletcher et al. (1907) proposing that lactate accumulation was the cause of reduced force production during fatigue. This has led to many conclusions being drawn about the exact role of lactate and it’s effect on muscular contractions. However, there is a lack of direct evidence linking lactate accumulation with muscle fatigue. Fitts and Holloszy (1976) demonstrated a significant inverse correlation ($r = -0.99$) between the rise in lactate concentration and the decrease in force production during stimulation of isolated muscle. This suggests that lactate does play a role in the fatigue process, but it does not indicate a mechanism, nor does it mean that there is a cause and effect relationship between lactate and decreased force production in skeletal muscle. Thus it is possible that lactate is not a contributing factor.

Lactate is a metabolite formed during glycolytic metabolism, which allows for the rapid production of adenosine triphosphate (ATP) from glycogen during intense exercise. It has been demonstrated that high lactate concentrations occur because of the inability of the mitochondria to utilize nicotinamide adenine dinucleotide (NADH). Under intense exercise conditions the muscle to converts pyruvate to lactate and converts NADH to NAD$^+$. This increase in lactate has been linked to the detrimental effects of fatigue on muscle function and could therefore cause the fatigue process in skeletal muscle.

It has been proposed by Williams and Klug (1995) that fatigue is due to an intrinsic mechanism built into the muscle that prevents the muscle cell from depleting itself of ATP and triggering irreversible injury. It is possible that lactate accumulation is an underlying factor in fatigue, and could be a trigger that ultimately prevents irreversible cell damage. It has been shown under certain conditions that as lactate concentrations rise in
the muscle, there is a subsequent decrease in force production (13). This suggests that lactate may be a signal that promotes fatigue in skeletal muscle. Based on this it does not seem at all illogical to trade a temporary inability to maintain force production for long term cell vitality (38). Therefore, fatigue could be a very important physiological mechanism that allows for continued use of the muscle after fatiguing contractions, no matter what the intensity which would depend upon on the person’s health.

The reduction in exercise tolerance because of fatigue may prevent individual’s from accomplishing everyday activities or reduce the ability of an athlete to compete, but it could vary well be a necessary occurrence. However, it is important to understand if there is a cause and effect relationship between the accumulation of lactate and fatigue. This could help to apply this knowledge in clinical settings in order to improve the quality of life for various individuals.

**Statement of Problem**

It has been known for many years that lactate concentrations increase as the intensity of exercise increases. An inverse correlation between the rise in lactate and the decrease in force production was first demonstrated by Fletcher et al. (1907). However, there are very few data demonstrating a cause and effect relationship between the increase lactate concentration and the decrease in force.

It is possible that since pH (- log [H+] ) decreases because of the increase in lactate, that the hydrogen ion is the contributing factor to the decrease in force and not lactate (12). In support of this notion are several studies linking the hydrogen ion and a reduction in force production under variety of conditions (10, 20, 22). However, most studies showing a negative effect of pH on muscle force production were performed below physiological temperatures (e.g. 20° C). Recently experiments conducted at near physiological temperatures (e.g. > 30° C) demonstrated that the effect of pH is minimal (40, 27) and in fact, in one study force production increased as pH decreased at 37° C (31). Therefore, acidosis alone may have little effect on tension production at physiological temperatures (31).

The overall objective of this investigation was to determine if lactate, independent of altered pH, effects muscle function. This was accomplished by examining the effects of lactate at low temperature where the effects of a decreased pH are noticeable and physiological temperatures where the effects of a low pH are small. Preliminary data demonstrated that increased lactate concentrations depressed force production and the rate of tension development (+dP/dt). This suggests that lactate may affect force by impairing Ca$^{2+}$ release from the sacroplasmic reticulum. Thus, a second goal was to determine the effect of lactate on Ca$^{2+}$ release and uptake in isolated sacroplasmic reticulum.
Significance of the study

Since there are few data directly implicating lactate’s role in the fatigue process, this study will help to validate or disprove any connection between lactate and fatigue. If this idea is clarified, then it may provided a better understanding to why muscle fatigues and possibly demonstrate a trigger point that causes the cascade of events resulting in fatigue. If this were the case it may be possible to design training programs to allow people to exercise at particular levels without producing lactate and triggering fatigue. This would be extremely useful in clinical environments, with patients where it has been demonstrated that exercise is beneficial in improving their quality of life. Alternatively, it could very well be that lactate is not important in the reduction of force production. Such a finding would also be valuable in that it could eliminate one more theory as to why a muscle fatigues.

Research Hypothesis

The following null hypothesis as tested during this investigation:

Ho₁: Tetanic force production of isolated whole EDL mouse muscle will not be reduced when exposed to increased concentrations of lactate.

Ho₂: The effects of lactate on tetanic force will not differ between 21° and 37° C.

Ho₃: The rate of Ca²⁺ release from the sacroplasmic reticulum isolated from mouse EDL muscle will not be reduced when exposed to elevated lactate concentrations.

Delimitations

The following delimitations set by the investigator:

1. The subjects were 40 adult, male Swiss-Webster mice.

2. Only one muscle was subjected to each condition (e.g. temperature or lactate)

3. Force production was represented by maximal tetanic contractions produced by the muscle.

Limitations

The following limitations were inherent in this experimental design:

1. The experiment will be performed in vitro which only mimics a true physiological environment.
2. The experiment was limited to temperatures of 21° C and 37° C.

3. The experiment only included one type of species (Swiss-Webster mice).

4. The experiment was limited to concentrations of 10, 20, 30, and 50 mM lactate

**Basic Assumptions**

The following basic assumptions were made by the investigator:

1. The mice were healthy during the study (e.g. the animals were obtained from virus-antibody-free commercial sources and observed by certified AALAS animal care technicians).

2. The muscles functioned properly at both temperatures (21° and 37° C).

3. There was no underlying factors within the muscle, unknown to the investigator, that would effect normal function.
Chapter 2:

Literature Review
Introduction

Skeletal muscle fatigue can be defined as a transient reduction in the ability of a muscle to produce an expected or desired force output (9). This phenomenon is observed in many places such as the work site, athletic events, or during everyday activities. It is also possible that the inability of the muscle to produce force involves a number of underlying factors such as cardiorespiratory or neuromuscular diseases (38). These diseases states are often associated with reduced time to exhaustion during muscular activity. Muscular fatigue not only occurs in individuals with diseases, but also healthy people. There are many underlying factors that have been attributed to fatigue, which can occur at a variety of sites in the body. However, no investigation has attributed a certain factor as being the single cause of fatigue. Thus, the purpose of this literature review will be to examine the possible role of lactate in force production during fatigue. It will focus on the formation of lactate, lactates association with fatigue, lactates effects on E-C coupling and the link between lactate and pH.

Mechanisms of Muscle Fatigue

Activation and contraction of muscle

Activation of the skeletal muscle originates from the brain and the spinal cord transmits a nerve impulse that then travels along a somatic motor neuron. This impulse, which is also called an action potential, travels towards the muscle. The action potential stimulates the motor nerve endplate to release acetylcholine from synaptic vesicles within the nerve, into the synaptic cleft where it binds to receptors located on the muscle membrane (sacrolemma). The binding of acetylcholine to its receptor causes another action potential to be formed along the sacrolemma, which then travels down the transverse tubules. The following events are commonly referred to as excitation-contraction coupling. When the action potential reaches the transverse tubule it triggers the ryanodine receptor, by unknown mechanisms, located on the terminal cisternae portion of the sarcoplasmic reticulum (SR), to release Ca$^{2+}$ through channels onto the sacroplasm. The Ca$^{2+}$ released then migrates to contractile apparatus, where it binds to troponin C. This causes the actin-myosin cross bridges to leave a weak binding state and enter a strong binding state. When this strong binding occurs the myosin-ATPase hydrolyzes ATP to ADP and Pi and energy. This release of energy causes contraction of the myosin head resulting in a pulling of actin over myosin and shortening of the muscle. By this process force is generated by the muscle (30). Attachment of new ATP allows the cross-bridge to detach and return to a weak binding state. The reserquesting of Ca$^{2+}$ by way of the Ca$^{2+}$-ATPase of the SR allows the muscle to relax and await another action potential. In this scheme, force output by the contractile apparatus is determined by the level of activation, i.e. the concentration of Ca$^{2+}$ present in the sacroplasm ([Ca$^{2+}$],) (37). The Fmax and the [Ca$^{2+}$]$_{50}$ of the contractile apparatus are defined by a sigmoidal relationship (force vs log Free Ca$^{2+}$) in that force production by the contractile apparatus is roughly proportional to myoplasmic free Ca$^{2+}$. The Fmax represents maximum force production by the contractile
apparatus and the \([\text{Ca}^{2+}]_{50}\) represents the concentration of \(\text{Ca}^{2+}\) required to elicit one-half (or 50%) of the maximal force production.

Many investigations have cited that failure one or more of these steps to be the cause of fatigue. For example, William and Klug (1995) propose that diminished SR \(\text{Ca}^{2+}\) release during fatigue, reduces contractile apparatus activation and force output. However, there are many steps that could potentially fail to prevent full activation or contraction of the muscle.

**Central fatigue vs peripheral fatigue**

Neuromuscular fatigue is classified as a failure of the transmission of an action potential to the muscle, therefore reducing the activation of contraction. This has been termed central fatigue. Many studies have shown that this unlikely to occur. Merton et al. (1950) demonstrated that when a muscle was fatigued by voluntary contractions, force production did not return with direct electrical stimulation of the muscle or stimulation of the nerve. This suggests that limiting factor does not occur within the nervous system, but within the muscle itself. This allowed the hypothesis to be formed that a transient change occurs within the muscle preventing force generation, hence the term peripheral fatigue.

**Mechanisms of peripheral fatigue**

An increasing amount evidence suggests that fatigue results from alterations in E-C coupling secondary to intracellular \(\text{Ca}^{2+}\) exchange (38). This is supported by four pieces of evidence, first, the intracellular levels of \(\text{Ca}^{2+}\) ([\(\text{Ca}^{2+}\)]\(_i\)) have been shown to decrease to approximately 50% of control levels during fatiguing stimulation (37). Second, it has been shown that by blocking the \(\text{Ca}^{2+}\) release channel with dantrolene sodium that force production may be reduced as is seen in fatigued muscle (23). Finally, application of caffeine, which evokes SR \(\text{Ca}^{2+}\) release, can restore force output of fatigued muscle. It is possible that during fatigue the functional properties of the SR are altered decreasing the peak myoplasmic \(\text{Ca}^{2+}\) concentration, and preventing \(\text{Ca}^{2+}\) saturation of Troponin C (TnC) on the contractile apparatus (38). If \(\text{Ca}^{2+}\) release is reduced it could prevent the contractile apparatus from being able to obtain \(\text{Fmax}\), therefore reducing whole muscle force. It is likely that the cause of fatigue is an intrinsic alteration in SR function which causes dysfunctions in E-C coupling and reduced force production.

Another theory that has been proposed is that the depletion of some substrate or the accumulation of a metabolite could prevent force development by the muscle. It has been demonstrated on numerous occasions that metabolites can have significant effects on E-C coupling events (11, 12). For example, the inorganic phosphate ion (Pi), has been shown to have detrimental effects on contractile apparatus function. Potma et al. (1995) demonstrated that with increasing concentrations of Pi, force produced by the contractile apparatus significantly declines. Thus an elevation in Pi during fatigue could cause force reduction. Another one of these metabolites is lactate or lactic acid. Numerous investigations have proposed that lactate accumulates during intense exercise (12). Some
studies have also demonstrated a significant inverse correlation between the rise in lactate concentration and the decline in force production (13). If lactate does depress force production, it should effect one or more steps in the E-C coupling process.

**Role of Lactate in Fatigue**

**Formation of lactate**

To ensure it’s viability, skeletal muscle must continually synthesize adenosine triphosphate (ATP). In addition, muscle requires ATP formation to allow for contraction and relaxation of the muscle. The muscle forms ATP, generally from glycogen, and then is used by the muscle as an energy source. Glycogen is considered the dominant substrate by which energy is created during high intensity activity (35). ATP can be formed from glycogen in several pathways including aerobic and anaerobic metabolism. The two differ in that aerobic metabolism requires O\(_2\) as an electron acceptor, while anaerobic metabolism operates without the need for O\(_2\). Anaerobic or glycolytic metabolism is a sequence of 10 enzymatically-driven reactions that degrades 1 mole of glucose from glycogen to pyruvate and forms 3 moles of ATP (35). Lactate is an end-product of glycolysis being formed from pyruvate with the conversion of NADH to NAD\(^+\).

During exercise, intracellular levels of lactate can accumulate in several ways. First, if the rate of glycolysis is rapid, then NADH production may exceed the ability of the mitochondria to shuttle the excess hydrogen ions. This results in pyruvate accepting the ‘unshuttled’ hydrogen ions causing lactate formation. Second, lactate dehydrogenase (LDH), catalyzes lactate formation from pyruvate. In fast fibers, the LDH isoform favors conversion of pyruvate to lactate. In slow fibers, the isoform favors the opposite reaction. During fatiguing intense activity, as fast fibers are recruited, lactate formation from pyruvate is accelerated. Third, a final explanation for lactate accumulation could be that the rate of removal is exceeded by the rate of production. This could be caused by the a reduction in the ability of the muscle to transport lactate across the sacrolemma and into blood stream. Therefore a rise in the concentration of lactate within the intracellular environment of the muscle may reflect limited lactate removal.

Any one or a combination of these mechanisms could cause the increase in muscle lactate concentration during fatigue.

**Accumulation of lactate during fatigue**

In 1847, J. Berzelius hypothesized that amount of free lactate in the muscle was proportional to extent that the muscle had been exercise. This led Fletcher et al. (1907) to propose that the increased [lactate] may play a role in muscle fatigue. The researchers established that lactic acid accumulated during repeated contractions of amphibian muscle. They found that the development of fatigue occurred within the same time frame as increasing concentrations of lactate ([lactate]). They also found that the muscle force production returned to normal as lactate within the muscle returned to normal. This led
Fletcher et al. (1907) to conclude that lactate was responsible for the decreased force production by the muscle. Later, similar results were produced by Fitts and Holloszy (1976), in which they found an increase in frog muscle [lactate] from 3.3 to 18.7 µmol/g with a subsequent decrease in force production of 36%. They also found a significant inverse correlation (r = -0.99) between lactate and force. This led Fitts and Holloszy (1976) to conclude that lactate must be responsible for some of the decrease in force production. However, since the force only declined by 36% after 15 minutes of stimulation, Fitts and Holloszy hypothesized that there must be a temporary, secondary mechanism that prevents the muscle from producing the expected force output.

Several other studies show correlations between lactate and force during fatigue. High correlations have been demonstrated in human exercise studies (18) and various isolated muscle studies (13). While correlations between the reduction in force and the rise in [lactate] have been shown, they do not imply a cause and effect relationship. This research demonstrates a causal link, but not a mechanism for how lactate interferes with muscle contraction, so it is possible that there is effect secondary to lactate accumulation causing the reduction in force.

**Conflicting views of lactate**

Hood et al. (1991) demonstrated that with repetitive stimulation (10 Hz) of rat gastrocnemius, lactate concentrations was elevated by 1 minute and force decreased to 40% of initial. However, with continued stimulation, lactate concentration returned to control levels, while tension remained depressed. This indicates that the role of lactate in muscle fatigue may not be significant, particularly during extended periods of contraction. If lactate is not present within the muscle during stimulation, it is difficult to determine it’s exact role in muscle fatigue.

**Effects of lactate on whole muscle force**

There are few studies that directly link lactate and fatigue (i.e. cause and effect relationship). It has been shown by Durkot et al. (1994) that by increasing the rate of pyruvate oxidation with dichloroacetate (DCA), thus reducing lactate concentrations, time to exhaustion is increased in exercising rats. It was established that by decreasing lactate production by 29%, rats were able to run on a treadmill for almost 68 minutes longer than the control animals. This presents direct evidence for lactate accumulation and the development of fatigue. It also should be noted however, that reduced production of lactate also spared glycogen stores, possibly by augmenting pyruvate utilization via the tricarboxylic (TCA) cycle. It has been postulated that lactate concentrations increase as a direct result of excessive pyruvate production exceeding the amount that can enter the TCA cycle (36). Therefore, it is quite possible that with increased concentrations of pyruvate during intense exercise, it is necessary to form lactate, which than may cause a subsequent reduction in the muscles ability to produce force.
In a more recent study, Hogan et al. (1995) found that lactate added at a normal pH to perfused working dog gastrocnemius muscle had no effect on initial force production, but it did accelerate the rate of fatigue in stimulated muscle. They found that increasing arterial lactate from 4.2 mM to 14.4 mM, muscle lactate increased from 8.1 mM to 12.0 mM. The apparent inhibition of muscle tension development with stimulation during the high lactate condition was alleviated as lactate was removed from the arterial blood after stimulation (17). It was also found that if lactate was reintroduced to the working muscle that force production fell by approximately 12% of the recovery tension. The investigators concluded that when there was an increase in arterial lactate at normal pH and an accompanying increase in muscle lactate, this resulted in decrease in developed tension by stimulated skeletal muscle independent of pH (17).

It must be pointed out that there is still no apparent mechanism for the role of elevated lactate and it did not cause a complete reduction in muscle force production. Therefore, recently investigators have sought to determine if lactate effects intracellular events in the muscle.

**The effects of lactate on E-C coupling**

If lactate accumulation contributes to the fatigue process, it should effect one or more of the E-C coupling events (i.e. Ca\(^{2+}\) release, Ca\(^{2+}\) activated force, etc.). Recently, it has been shown that impairment of the SR Ca\(^{2+}\) release channel is a relevant factor in the reduction of force during fatigue (11). Inadequate availability of Ca\(^{2+}\) to the myofilaments of the muscle could be caused by some blocking or transient modification of the SR Ca\(^{2+}\) release channel (38). Favero et al. (1995) recently showed increasing lactate from 2 to 20 mM inhibited Ca\(^{2+}\) release from isolated SR vesicles. Also, lactate inhibited [\(^3\)H]ryanodine binding to SR vesicles and decreased the single channel open probability. The researchers found that in the presence of high lactate the [\(^3\)H] ryanodine binding was reduced from 3.2 pmol/mg to 1.5 pmol/mg. Ryanodine binds with nanomolar affinity to open SR Ca\(^{2+}\) channels. This finding that [\(^3\)H] ryanodine binding was depressed suggests that lactate causes fewer channels to respond to activating stimuli (11). The effects on a single release channel was examined in a lipid bilayer membrane. The open probability of SR release channel was reduced by approximately 75% when it was in a high lactate environment (20mM). It was found that normal gating behavior of the channel was inhibited in the presence of lactate, so that the open probability was decreased(11). If the release channel probability of opening or the rate of release is reduced in vivo then is possible that not enough Ca\(^{2+}\) will be released during tetanic contraction to elicit Fmax of the contractile apparatus. The results of Favero et al. (1995) suggest that with increased lactate there is a decrease in the responsiveness of the SR Ca\(^{2+}\) release channel. This may, in turn, promote a decline in force production by disrupting E-C coupling and may contribute to muscle fatigue (11). These results provide a possible mechanism for reduced force production when lactate is produced by the muscle.

It has also been proposed that at the onset of fatigue, the Fmax of the contractile apparatus is directly affected and responsible for a part of the loss of force generation.
This could be caused by a reduction in the force produced by the cross bridges or to a decline in the fraction of cross bridges in the force generating state. The lactate anion may have a detrimental effect upon either factor, such that $F_{max}$ or in $Ca^{2+}$ sensitivity of the contractile apparatus are reduced. Andrews et al. (1996) showed that lactate directly effects the contractile apparatus of chemically skinned rabbit psoas fibers, contributing to the loss of force generation. They found a significant 7% reduction in $F_{max}$ of the muscle when the in presence of 25 mM lactate at a pH 7.0 (2). However, it seems that this effect is concentration specific, since there was no effect in concentrations of 10 and 50 mM. Chase and Kushmerick (1988) also found that a lactate concentration of 50 mM had no effect on the contractile apparatus. The control of the pH allowed Andrews et al. (1996) to conclude that the reduction was not be caused by the $H^+$, but in fact, the lactate anion. Lactate was also found to shift the force vs. pCa relationship of the contractile apparatus, decreasing the slope while leaving the $Ca_{50}$ unchanged. This means that $Ca^{2+}$ sensitivity was increased at low $Ca^{2+}$ concentrations and decreased at higher concentrations (2). This could result in a slowed rate of relaxation of the muscle that is apparent during fatigue (2). These results represents another possible mechanism for lactate effecting force production in the intracellular environment of the muscle.

The above studies seem to indicate that lactate may influence force production during fatigue. It was shown that lactate effected two different steps in the E-C coupling process independent of a reduced pH. These are force production by the contractile apparatus and $Ca^{2+}$ release by the SR. In either case, the alteration could result in diminished whole muscle force and the development of fatigue.

**Lactate and Hydrogen Ions**

*Lactate accumulation and changes in pH*

An alternative mechanism by which lactate production may effect force during fatigue is through the action of the $H^+$. Fitts (1994) postulated that the high inverse correlation between lactate and force is, for the most part, dependent on the high correlation between lactate and free $H^+$. He suggested that the force depressing agent is the $H^+$ and not lactate. Therefore, increased [H$^+$] associated with increased [lactate] during fatigue presents another possibility for reduction in force. At a physiological pH of 7.2, 99.5% of lactate disassociates into a lactate anion and a $H^+$ cation (15). With an increase in [H$^+$], there is a subsequent drop in pH, which has been shown to have a role in muscle fatigue. The intracellular pH of mammalian skeletal muscle has been shown to fall from 7.0 to as low as 6.2 during fatigue (12). With this disassociation of lactate and increase in [H$^+$], impairment on various steps in the E-C coupling process may occur.

It has also been shown that in order for extracellular lactate to passively cross the sacrolemma it may have to be actively coupled with a $H^+$ (24). This creates a problem with studies in which extracellular lactate is manipulated, in that if intracellular concentrations of lactate rise, a drop in intracellular pH also occurs. This complicates the study of the
effects of extracellular lactate on whole muscle force because it is difficult to discern if the effect is caused by the lactate anion or by H⁺.

**H⁺, muscle force production, and E-C coupling**

Adverse effects of reduced pH on the intracellular environment of the muscle have been widely demonstrated and reproduced. A decline in pH has been shown to influence functioning of both the SR and the contractile apparatus. Cooke et al. (1987) suggest that the slowed relaxation of the skeletal muscle exhibited during fatigue is caused by a reduction in the rate of SR Ca²⁺-ATPase activity due to a low intracellular pH (pHᵢ). They showed at a pHᵢ of 6.4 that Ca²⁺-ATPase was reduced by 25-30%. This suggests that if there is slowed clearance of Ca²⁺ from the cytosol by the SR relaxation rate would be depressed. This notion was supported by Baker et al. (1995) who demonstrated that the rate of relaxation of frog muscle increased by 68% at a pHᵢ of 6.3. It has been suggested that the H⁺ ion competes for Ca²⁺ binding site of the ATPase which would reduce the rate of clearance from the cytosol (3). It is also possible that reduced rate of Ca²⁺ uptake could prevent the SR from being fully loaded with Ca²⁺, such that an insufficient amount of Ca²⁺ is available for subsequent release to create a forceful contraction.

Ma et al. (1988) found in isolated SR that a pH of 6.4 diminished the frequency and duration of Ca²⁺ release channel openings. This was confirmed by Williams and Ward (1992) who have shown that at a reduced pH (6.5), the rate of Ca²⁺ release is inhibited by 44%. Also, it was shown by Rousseau and Pinkos (1989) that a reduced pH resulted in a decrease in the open probability of the release channel. This suggests that the channel may close prematurely and prevent Ca²⁺ release from the SR. If the [Ca²⁺]ᵢ is decreased by diminished Ca²⁺ release secondary to a reduction in intracellular pH then it could result in diminished myofilament activation and reduced force output (38).

In contrast to the above, Lamb et al. (1992) reported that a pH of 6.2 had no effect on the Ca²⁺ release in skinned fibers, and they postulated that the decline in force at the low pH came from direct inhibition of the contractile apparatus. Therefore, some controversy exists on whether or not increases in H⁺ concentration during fatigue interfere with SR function. However, the majority of studies point to depressed SR Ca²⁺ uptake and release at a low pH.

The H⁺ has also been shown to affect the myofilaments of striated muscle. Robertson and Kerrick (1979) has shown that a decrease in pH could cause competition for binding between H⁺ and Ca²⁺ on troponin C (TnC). They found that there was rightward shift of force-pCa curve, requiring a higher [Ca²⁺] to elicit activation of the muscle at a pH 6.5 than at a pH 7.0. This suggests that protons may alter the pCa-force relationship by preventing Ca²⁺ from reaching the activating sites on the TnC (33). This effect of the Ca²⁺-sensitivity has also been demonstrated by Lynch and Williams (1994). However, they propose that the shift could be caused by a inhibition of myosin ATPase activity, which would lead to decrease in cross-bridge cycling and maximal force generation (21). This notion was confirmed by Potma et al. (1995), who found that a
20% decrease in the myosin ATPase activity at a pH of 6.4. Since myosin ATPase is
determined, in part, by the kinetics of crossbridge detachment, this suggests that cross-
bridge cycling is affected by H⁺. Thus, H⁺ decreases Ca²⁺ sensitivity by possibly altering
Ca²⁺ binding to TnC and/or by slowing cross bridge cycling.

The H⁺ also has significant effect on the maximal velocity of shortening (Vmax) by
the contractile apparatus. Cooke et al. (1987) showed that a pH of 6.0 reduced the Vmax
by 25-30%. This suggest that at a decreased pH it takes longer for a muscle to produce a
maximum contraction, which indicates a possible reduction in the number of active cross
bridges.

Finally, it has been demonstrated by Fabiato and Fabiato (1977) that a reduction in
pH may have no effect on the maximum Ca²⁺-activated force. However, it was shown by
a number of groups that a reduction in pH significantly reduced maximum Ca²⁺ activated
force (16, 20, 26). Therefore, the results of a reduced pH on the contractile apparatus are
conflicting, but generally show that there is a depressant effect of an increased [H⁺] in that
Ca²⁺ sensitivity, Fmax and Vmax are reduced.

Influence of temperature

It should be noted that a reduction in intracellular pH may not be the only
contribution factor to fatigue. Renaud et al. (1986) demonstrated that a reduction in pH,
from 7.0 to 6.2 reduced tetanic force by less than 40%. However, during fatigue, tetanic
force decreased by 70% while pH, was reduced to 6.4. Thus, the effect of intracellular
acidosis was much too small to account for the large suppression seen following fatiguing
stimulation (32). This was also demonstrated by Adams et al. (1991), who found that
reduction in intracellular pH had no effect on peak tetanic force in hypercapnic infused cat
muscle. Also the change in pH did not consistently correlate with changes in peak tetanic
force. This suggests that a decrease in pH, during repetitive stimulation cannot be a single
cause of fatigue and there must be some other effect on contractile activity (32).

A confounding factor of many studies which have investigated pH effects on
skeletal muscle function is the experimental temperature. Most early studies were done at
≤ 22°C rather than at temperatures close to physiological (e.g. 37°C). Ranatunga
(1987), showed that at temperatures closer to physiological, a reduction pH did not result
in a reduction in force in whole muscle fibers. He confirmed that there was significant
reduction in force at a pH of 6.5 when the temperature was maintained at 15°C.
However, he also showed that force production was actually higher (~ 40%) at 30°C,
compared to 15°C which was still depressed, when the pH was reduced to 6.5 ( ).
Therefore, the effect of pH on the muscle could be temperature dependent. It is possible
that acidosis may not have an adverse effect on tension development at temperatures
similar to physiological.

The results of Ranatunga (1987) were confirmed by Wiseman et al. (1996). It was
shown that the force inhibition during hypercapnea was greater at 15°C than at 25°C. It
was therefore concluded that changes in intracellular pH may have little or no role in decreased force production during fatigue in whole muscle.

A similar temperature dependence was found in skinned fibers by Pate et al. (1995), who used high levels of CO$_2$ to reduce pH. The investigators were able to show that compared to pH 7.0 Fmax was reduced at pH 6.2 at 15° C. However there was no reduction in Fmax at pH 6.2 when temperature was elevated to 30° C by a temperature ‘jump’ method. This contradicts the earlier stated studies in when force was lowered at decreased pH levels using skinned fibers. This may indicate that the H$^+$ may have little effect on the contractile apparatus during fatigue at physiological temperatures.

Many conclusions have been drawn about the role of a decreased pH in fatigue of skeletal muscle. Many studies have shown various disruptions in E-C coupling and whole muscle force production when the pH was reduced. However it has been shown that these disruptions are unlikely to be cause of fatigue. When physiological temperatures are compared with reductions in pH, it seems possible that a the H$^+$ may not play a role in the fatigue process.

**Summary and Conclusions**

It seems that there is often a strong relationship between lactate accumulation and decreased force production during fatigue. This has been demonstrated in both human and animal studies. Each exhibited a significant inverse correlation between force and lactate concentration during fatigue (13, 18). Based on the work with isolated SR and skinned fibers, lactate may depress force by disrupting events in the E-C coupling process. These provide a possible mechanism for lactate’s role in the fatigue process. However, work with lactate exposure in whole muscle has yielded inconclusive results.

A complicating factor in these studies is the reduction in pH$_i$ that accompanies the increase in lactate extracellular concentrations. This may compound the role of lactate in the fatigue process because it provides multiple effects (i.e. lactate anion and hydrogen ion) for the decrease in force production. The increase in H$^+$ concentration may also decrease force by affecting various pathways involved in E-C coupling. However, it should be emphasized that the reduction in pH seems to be relevant at temperatures well below physiological (< 22° C). Thus, it is unclear if lactate affects force output of whole muscle by direct action or secondary to the decrease in pH.

It is possible that important insight into the effects of lactate on skeletal muscle force output may be gained by examining its effects at low temperatures and at physiological temperatures. At low temperatures (≤ 22° C) the reduction in pH$_i$ may be important, whereas at physiological temperatures (37° C) it may be negligible. Thus, a depression in force by lactate at both high and low temperatures would suggest a direct effect of lactate on muscle force. Conversely, an effect of lactate on force at ≤ 22° C but not at 37° might indicate that any adverse effect is due to the declines in pH$_i$. These results would allow for possible conclusions to be drawn about the effects of the lactate
anion, independent of pH, on muscle function during fatigue. Since there are few studies identifying lactate's role in fatigue, this would help provide a theory to lactate's involvement in skeletal muscle fatigue.
Chapter 3:

Methodology
Research Design and Methods

In Vitro Muscle Preparation:

The animal research protocol for this project was approved by the Virginia Tech Animal Care and Use Committee. Whole extensor digitorum longus muscles (EDL) were harvested from adult male Swiss-Webster mice. All mice weighed approximately 30 gm. Each mouse was anesthetized with sodium pentobarbital (50 mg/kg, IP). Each animal was placed in prone position and determined to be properly anesthetized with the toe pinch technique. The skin was removed from the distal portion of the fibula to the proximal area of the tibia using rat toothed forceps and scissors. The tibialis anterior muscle was first removed with careful cutting from the distal portion of the tendon along the tibia to the proximal tendon. The EDL was then isolated and 3-0 silk suture was secured to each furthermost portion of the tendons. The muscle was carefully removed from the animal by cutting the untied tendons. This procedure was carefully repeated in the other limb. The animal was then euthanized with an overdose of pentobarbital (IP injection).

Muscles were mounted in a temperature controlled chamber filled with the Ringer solution. The whole muscle Ringer solution consisted of (in mM): 135 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 1 Na$_2$HPO$_4$, and 15 NaHCO$_3$. The solution was bubbled continuously with 95% O$_2$ and 5% CO$_2$ and maintained at a pH of 7.2. The lactate was added from a stock solution (5000mM) to the above Ringer solution. One end of the muscle was attached to fixed post and the other to an isometric force transducer (Grass FT03). Tetanic contractions were evoked by trains of electrical pulses (100Hz, 250ms) delivered across ring electrodes placed at either end of the muscle (Grass S88). Temperatures of 21° and 37° C were maintained via a circulating water bath. Prior to each experiment, muscles were allowed to equilibrate for 15 minutes and length adjusted to obtain maximal tetanic force.

For the first three minutes the muscle was exposed to control conditions (i.e. no lactate). At three minute intervals, lactate (final concentration of 10, 20, 30 and 50mM) was added directly to muscle bath from the stock solution. During each three minutes interval tetanic contractions were evoked every 30 seconds. The control muscles underwent the same protocol except no lactate was added to the bath. The muscles were then weighed, length measured and computed for cross sectional area. The muscle was then removed and properly disposed.

Signals from the force transducer were digitized (MetraByte DAS 1608, 12 bit A/D) at 1000 Hz and analyzed for peak developed force ($P_o$) as well as the peak rates of force increase ($+\text{d}P/\text{d}t$) and decrease ($-\text{d}P/\text{d}t$).
Measurement of Ca\(^{2+}\) Release and Uptake

The homogenizing buffer consisted of 250mM sucrose, 20mM N-2-hydroxyethylpiperazine-N' -ethanesulphonic acid (HEPES, pH 7.5), 2% sodium azide (NaN\(_3\)), and 0.2mM phenylmethylsulfonyl flouride.

EDL muscles were harvested from mice as described above. Muscles were removed and trimmed of connective tissue and then placed in a ice cold homogenizing buffer. They were then miniced with scissors and homogenized in 20 volumes or 200µL (whichever was greater) of cold buffer using a 5mm probe (Pro 200) and three 15 second bursts. The crude homogenates were then centrifuged at 1,600g for 10 minutes (2° C). Following centrifugation, the supernatant fraction was removed and stored at -80° C. Total protein concentration was determined with the Bradford protocol by Bio-Rad.

The assay buffer consisted of 100mM KCl, 20mM HEPES, 7.5mM sodium pyrophosphate 2µM free Ca\(^{2+}\) and 0.5mM free Mg\(^{2+}\) (pH 7.0, 37° C). Ca\(^{2+}\) uptake and release were measured by adding 125µg homogenate supernate to 1ml of the assay buffer. After 2-3 minutes of equilibration, Ca\(^{2+}\) uptake was initiated by the addition of 0.5mM of MgATP and continued until no change was observed in extravesicular free Ca\(^{2+}\). After uptake was complete, lactate (25mM) was added from stock and the preparation was allowed to equilibrate for two minutes. Ca\(^{2+}\) release was then initiated by 25µM of AgNO\(_3\). MgATP, lactate, and AgNO\(_3\) were added by Hamilton syringe.

Measurement of Ca\(^{2+}\) uptake and release were performed using a JASCO CAF-110 flurometer and Indo-1 as the extravesicular free- Ca\(^{2+}\) indicator. Excitation light came from a xenon pressure lamp (75W) equipped with a monochromator that contained a 349 nm interference filter. Emission fluorescence was determined by two photo multipliers using 500 nm and 410 filters. The ratio of the emission fluorescence at 500 and 410nm was sampled via a microcomputer (2 Hz) and converted into free Ca\(^{2+}\) concentrations using the following equation: \([Ca^{2+}]_{force} = K_d \cdot B \cdot (R - R_{min}) \cdot (R_{max} - R)^{-1}\), where \(K_d\) is the dissociation constant for Ca\(^{2+}\) and Indo-1 (200 nm), \(B\) was the ratio of fluorescence measured at 500 nm in the presence of zero and 100µM free Ca\(^{2+}\). \(R_{min}\) and \(R_{max}\) represented fluorescence ratios in the presence of zero and 100µM free Ca\(^{2+}\). The rates of Ca\(^{2+}\) release and uptake were computed from the steepest positive and negative slopes of the free Ca\(^{2+}\) curve versus time curve and normalized by [protein].

Data Analysis:

All data were collected by computer for subsequent analysis. Data was analyzed statistically with the Sigma Stat package, with a significance set at p< 0.05. Interactions between time and condition in the whole muscle were examined by a two-way repeated measures ANOVA. A Newman Keuls post-hoc was used to determine interactions when effects were noticeable. Differences between release and uptake rates in isolated SR were analyzed using a one-way ANOVA.