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Longissimus muscle fatigue and injury response due to electrical stimulation with varied work/rest ratios

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LONGISSIMUS MUSCLE FATIGUE AND INJURY RESPONSE DUE TO ELECTRICAL STIMULATION WITH VARIED WORK/REST RATIOS

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CHAPTER 1 – INTRODUCTION

1.1 Epidemiology

Every day 6307 people in the United States sustain a lost-time work injury or illness (Schulte, 2005). This represents a $140 billion annual cost to society, in the form of medical and emergency services, employee and employer productivity losses, and lost quality of life (Miller and Galbraith, 1995). To put that into perspective, the costs of workplace injuries and illnesses are greater than the expenditures for Acquired Immune Deficiency Syndrome (AIDS), Alzheimer's disease, and arthritis, and similar to cancer and heart and stroke diseases (Leigh, 2000). Despite the larger costs associated with occupational injuries and illnesses, the aforementioned diseases garner far more public attention and resources (Leigh et al., 1997).

A small percentage of workers do not resume their duties soon after the injurious event (Turner et al., 2004). It is this small percentage of people that contribute to most of the cost of occupational injury and illness (Hashemi et al., 1997). However, the workers that do return quickly may experience recurrence of injuries, particularly if exposed to the same risk factors, and these recurrences result in increased costs (Wasiak et al., 2006). In addition there are workers that have experienced an injury but the injury is not serious enough to miss work. These employees will often have reduced productivity while working due to the injury (Meerding et al., 2005).

Workplace injuries and illnesses by definition consist of the two categories: illnesses and injuries. An example of an occupational illness is chronic exposure to chemicals resulting in respiratory problems. Injuries can be

categorized as physical in nature, such as those occurring to the musculoskeletal system. Injuries will be the focus of this study, as they comprise approximately 95% of all non-fatal occupational injuries and illnesses, with the remaining 5% attributable to illnesses (Bureau of Labor Statistics, 2009b).

The largest contributor to physical workplace injuries are musculoskeletal (Punnett and Wegman, 2004). Sprains and strains account for the largest number of musculoskeletal workplace injuries, 39% of the total, with the largest event (45%) being the result of overexertion (Bureau of Labor Statistics, 2009a). These types of injuries are commonly referred to as musculoskeletal disorders (MSDs) or injuries that can be addressed through the science of ergonomics (Bureau of Labor Statistics, 2009a). In Washington State between the years 1990-1998, approximately one-fourth of the compensation claims were due to MSDs with the average cost per claim exceeding all other claims (Silverstein et al., 2002).

The low back is consistently the most frequently injured body part (Silverstein et al., 2002), contributing to 40% of the strain and sprain injuries (Bureau of Labor Statistics, 2009a) (figure 1-1). One of the most common diagnoses of back injury is back strain (Keyserling, 2000). Overexertion due to lifting (in which the low back is primarily involved) contributes to a large proportion of the MSDs (Bureau of Labor Statistics, 2009a).

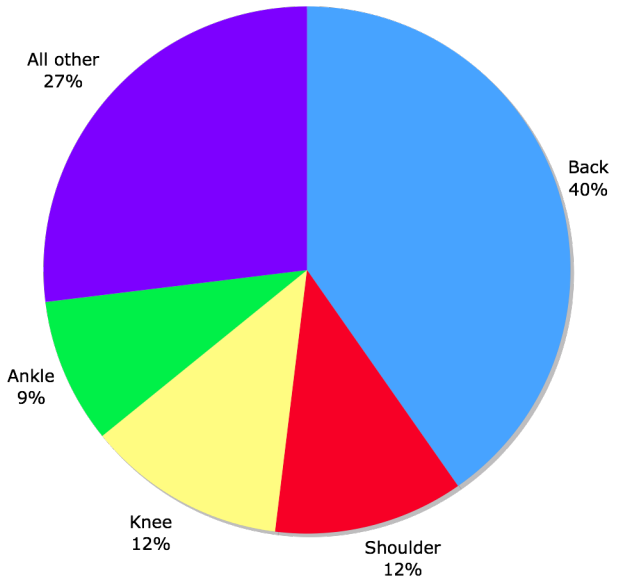


Figure 1-1: Occupational sprains and strains by body part (Bureau of Labor Statistics, 2009a)

1.2 Low Back Injury Risk Factors

A model developed by the National Institute of Safety and Health (NIOSH) determines the acceptable load to be lifted due to the amount of compressive force on the lumbar spine. Based on experiments performed on cadaveric sections of the spinal column, the committee that developed the NIOSH lifting equation selected 3400N of compressive force as the biomechanical injury risk criterion (Waters et al., 1993). In these experiments, a compressive load was placed on the spinal unit, consisting of a vertebral disc between two vertebrae, and the amount of load to cause damage was recorded. The observed injury from this mechanism was vertebral end plate fracture (Dolan and Adams, 2001).

The NIOSH equation developed in 1981 and revised in 1991 (Waters et al., 1993) addresses the risk factors of lifting. Factors within the model include

the following variables: horizontal distance of the lift, lift height, lift distance, frequency, and duration. The output is a prediction of the amount of weight that is acceptable to lift.

In typical lifting situations, in addition to the weight of the object, the erector spinae muscles contribute to the compression force. The muscles of the spine, collectively known as the erector spinae, consist of the longissimus thoracis and the iliocostalis (figure 1-2). These muscles, located adjacent to and along both sides of the spinal column, together with the multifidus control the anterior saggital rotation of the lumbar spine (Bogduk, 2005). The erector spinae muscles are the primary movers during repetitive lifting tasks (Kim and Chung, 1995). These are the muscles that extend the spine from a flexed posture and maintain the spine in an upright posture throughout the workday.

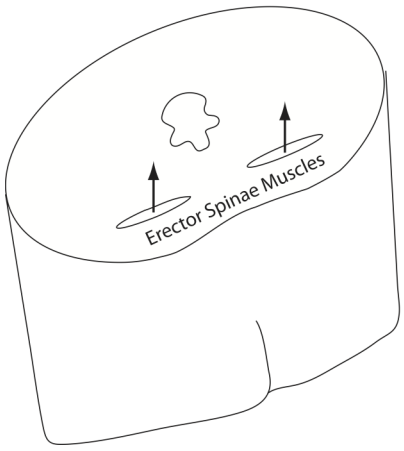


Figure 1-2: Low back anatomy and force estimation.

Contraction of the erector spinae muscles increases disc pressure due to the compressive forces of these muscles (Bogduk, 2005). This is particularly true when lifting loads a large horizontal distance from the spine. This creates a large moment around the lumbar spine due to the large distance from the spine

to the load and the short moment arm from the erector spinae to the spine. It is thus not surprising that the modifying variable with the greatest effect on the acceptable load in the NIOSH equation is the horizontal distance of the load to the spine.

Increasing horizontal distance of the load can increase the amount of spine flexion. Flexion of the spine results in an increased number of endplate fractures compared to neutral postures in a cadaveric model (Gallagher et al., 2005). Thus, forward flexion is a critical factor in predicting low back injury (Keyserling, 2000). It is also a factor in producing posterior disc herniation (Adams and Hutton, 1985; McGill, 2004).

Flexing the spine when lifting a load is unavoidable (Dolan et al., 1994). One study found axial compression on the spine to be a poor predictor of low back injury (Granata and Marras, 1999). Flexed low back postures are found to increase the amount of shear force acting on the spine and shear force has been postulated to be a better determinant of low back injury than compression (McGill, 2004). One advantage of flexing the lumbar spine is the unloading of the zygapophyseal joints, as increased damage results in these joints in neutral posture and not in flexed posture (Gallagher et al., 2005; Yang and King, 1984).

Increased compression due to muscle contraction provides stability to the spine (Bonato et al., 2003). Anecdotally, people have injured their backs when bending over to pick up very light loads. In these situations the amount of compression to the spine is well below the NIOSH limit of 3400N. The mechanism of injury in these cases may be due to a lack of compression,

contributing to decreased spine stability. Lack of proprioceptive feedback has been proposed as a reason for decreased stability leading to low back injury (Pope et al., 2002).

Cholewicki and McGill (1996) provided a model depicting a continuum of injury based on spine stability (figure 1-3). They suggested that there is an optimal range of compression where injury is less likely to occur (Cholewicki and McGill, 1996). At low compression the spine is unstable and can be subject to an instability injury, while at high compression the spine can be injured due to the loads on the spine.

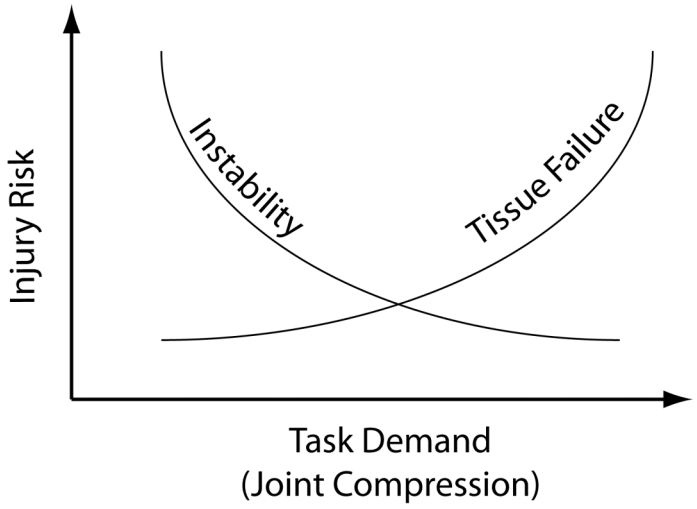


Figure 1-3: Spine instability model (Cholewicki and McGill, 1996).

The approach of using models, such as the NIOSH lifting equation, has been the primary low back risk evaluation methods in the field of ergonomics. These models are restricted to static or quasi-static saggital symmetric lifting settings (Marras et al., 1995). In industry, lifting can be dynamic with a range of velocities and accelerations of lifts. Marras et al (1995) developed an evaluation method that takes into account the three-dimensional aspects of lifting. They

suggested that risk of low back injuries is a combination of: lift rate, lateral and twisting motions, trunk flexion angle, and external moment (Marras et al., 1995).

In many instances workers are required to lift loads many times throughout the day. This frequent lifting can result in failure due to fatigue of vertebral endplates, vertebral bodies, and zygapophyseal joints, as evidenced in a cadaveric study (Gallagher et al., 2005). Spinal segments have been repetitively tested and fatigue damage occurred at forces found during normal daily activity (Hansson et al., 1987). Lifting frequency has an inverse relationship with load as a large number of cycles with low load can induce injury (McGill, 2004). Thus, lifting frequency is a primary risk factor for low back disorders (Bonato et al., 2003).

Repetitive lumbar spine flexion can have an effect on the spinal ligaments. The ligaments of the lumbar spine can experience creep due to the repeated flexion, affecting the stability of the lumbar spine. Repeated flexion for 20 minutes in a cat model induced lumbar spine muscle spasms that were still present the next day (Claude et al., 2003). It has been proposed that the elicited spasms are a protective mechanism to restore stability (Solomonow, 2004).

Psychophysical analysis is another method to evaluate risk of low back injuries due to lifting and it is more appropriate to use in frequent tasks (Keyserling, 2000). Psychophysics is the relationship between a physical stimulus and the person's perception of that stimulus. In the case of lifting, it is the relationship between the load lifted and people's perception of that load that is being lifted.

Utilizing psychophysics, tables have been developed to determine maximal acceptable weights of lift, carry, push, and pull (Snook and Ciriello, 1991). Simulating a task was carried out and the worker was asked how much load could be lifted in that task scenario for an 8-hour day. The workers would be allowed to adjust the load but not the parameters of the lift such as box size, horizontal and vertical distance, or the lift frequencies. These tables have been useful for determining the acceptable loads for various repetitive tasks.

Another method for incorporating frequency into acceptable task demands has been to determine the amount of energy expenditure required for a task. Regression equations have been developed for various task scenarios to predict amount of energy expenditure (Garg et al., 1978). Based on this result, a determination as to the acceptability of the load could be made. The NIOSH equation also bases their formula in part on energy expenditure. They suggested a limit of 9.5 kcal/min as acceptable for 50% of the population (Waters et al., 1993). However, while lifting guidelines incorporate repetition, they may not be specific enough to the fatigue experienced by the low back (Gallagher et al., 2005).

High force, awkward postures, and increased frequency, summarize the risk factors for low back injuries in the workplace. Each of these variables is addressed in several evaluation tools used to predict the risk of injury. Unfortunately, the assessment tools may not be addressing all the variables involved in workplace MSDs, as the percentage of MSDs (29%) out of the total

lost time injury and illness cases has remained relatively constant since 2005 (Bureau of Labor Statistics, 2009a).

1.3 Work/Rest Ratios

Local muscle fatigue may play a role in the determination of MSDs (Armstrong et al., 1993). Researchers have measured local muscle fatigue in situations that placed volunteers in conditions that would theoretically have an increased risk of injury. For example, performing assembly tasks in two different postures found evidence of increased fatigue in the more demanding posture (Bosch et al., 2009). Also, cyclic lifting, a risk factor for MSDs, resulted in local muscle fatigue (Bonato et al., 2003).

Surface electromyography (EMG) is a commonly used tool to measure local muscle fatigue (Cifrek et al., 2009). These physiological recordings are typically performed in short-term experiments, and the relationship to chronic effects is unclear (Mathiassen, 1993). Unlike acute injuries, in which the injurious event is due to a one-time force applied to the musculoskeletal system, chronic injuries due to fatigue are the result of repetitive movements.

Figure 1-4 provides a theoretical explanation for the development of chronic injuries (Barr and Barbe, 2004). The capacity of the tissue is initially able to handle the task. However, when a muscle fatigues, it cannot maintain the required power output (Fitts, 1994). With rest, the tolerance increases but not necessarily up to the original capacity. Eventually, without appropriate rest, the demand will exceed the capacity and result in injury. Unfortunately, rest periods are not typically under the control of the worker, resulting in the inability to relieve musculoskeletal discomfort until a scheduled work break (Punnett and Wegman,

2004). Thus, while a specific relationship between local muscle fatigue and injury is not well defined, it can be assumed that local muscle fatigue can be used as an indicator of injury risk (Nussbaum et al., 2001).

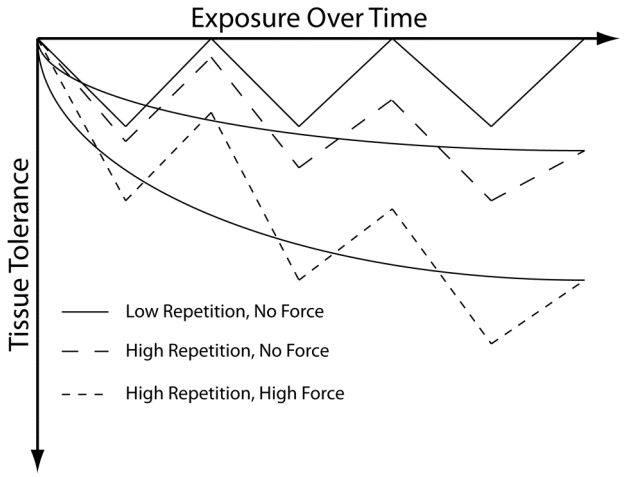


Figure 1-4: Theoretical explanation for the development of chronic injuries (Barr and Barbe, 2004)

Providing rest breaks seems to be an effective strategy in ameliorating the effects of fatigue. Sundelin (1993) had two groups of volunteers perform a repetitive work task. One group was provided rest breaks, while the other was not. The group with rest breaks had the same amount of productivity as the group without breaks; thus, their work pace was increased to match the productivity. It was found that the group provided with rest breaks showed less signs of muscle fatigue, even though their work pace was higher (Sundelin, 1993).

Rest breaks may be detrimental if productivity is not held constant. Studies have found that while increased endurance is achieved due to rest breaks, the EMG results indicated more fatigue in these muscles (Bystrom et al., 1991; Mathiassen, 1993). The rest breaks allowed the volunteers to continue the tasks for a longer period of time than those without breaks, which increased the

exposure to the task. Limiting the amount of exposure while providing rest breaks can be an effective method in reducing fatigue (Mathiassen and Winkel, 1996; Mathiassen and Winkel, 1991).

The amount of rest allowance required is dependent on the force and duration of the muscular contraction (Rohmert, 1973). Figure 1-5 displays the results of an experiment in which isometric contractions were held at varying percent maximum voluntary contraction (MVC). The longer the contraction is held and the higher percent MVC, the more rest allowance is required. Thus, a repetitive job can be defined by the amount of required force, contraction duration, and rest duration (Wood et al., 1997).

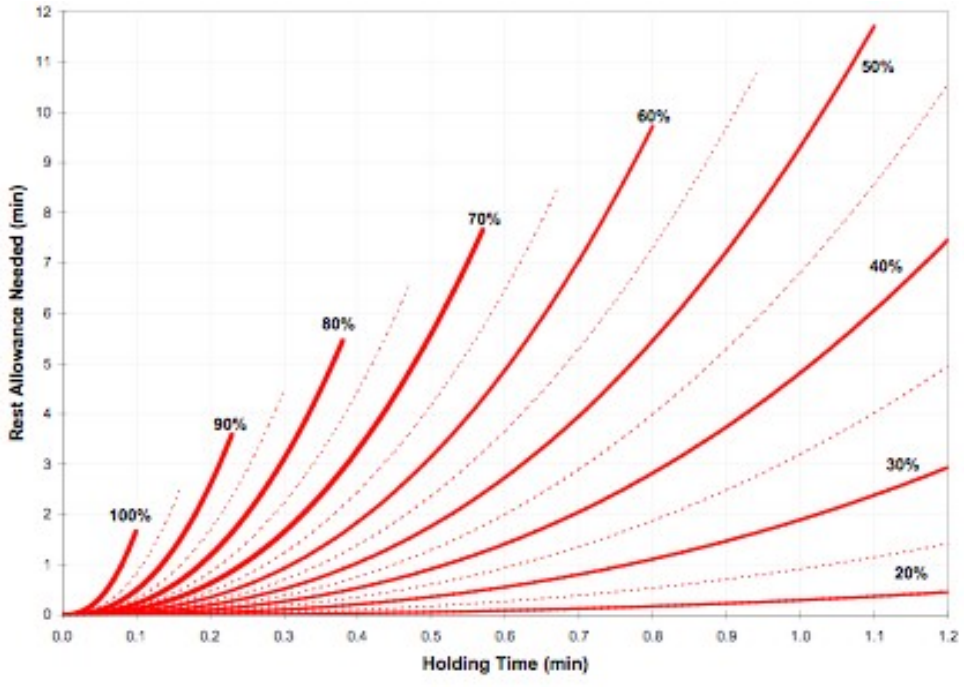


Figure 1-5: Rest allowance prediction (Rohmert, 1973)

This combination of contraction time and rest time is defined as cycle time. A general range of cycle times observed in industry is 20-180 seconds (Iridiastadi

and Nussbaum, 2006). A relationship between the contraction time and cycle time can then be established, known as duty cycle, which is the ratio of contraction time to cycle time. For example, a cycle time of 20 seconds, with 10 seconds of contraction time and 10 seconds of rest time, represents a duty cycle of 0.5 or 50%. Mathiassen and Winkel (1991) suggested that the parameters of mean load, duty cycle, and cycle time provide a description of a repetitive task.

In general, higher duty cycles result in greater fatigue (Nussbaum et al., 2001). This is logical as, by definition, higher duty cycles have less rest time. A study in which the supraspinous ligament in a cat was cyclically stretched suggested that a 50% duty cycle can avoid injury (Courville et al., 2005). Also, shorter cycle times may reduce these fatigue effects (Iridiastadi and Nussbaum, 2006).

1.4 Low Force Prolonged Contractions

An explanation for the occurrence of muscle fatigue and injury may be found in the morphology of muscle. Muscles are composed of many fibers and the fibers can differ in terms of their properties. Specifically, muscle fibers can be separated based on their energy metabolism.

Type I fibers utilize oxidative phosphorylation for their energy requirements. Although this results in slow activation, these fibers are generally fatigue resistant, due to the abundance of energy available, in the form of oxygen. Conversely, type II fibers fatigue quickly due to their reliance on the glycolytic system for their energy requirements. These fibers are fast activating and can generate a larger amount of force than the slow twitch type I fibers. Fast twitch fibers can be further differentiated into type IIA and IIB. Type IIB fibers are

faster and easier to fatigue, due to their high glycolytic dependence, whereas type IIA have a mixture of oxidative and glycolytic capacity (Kraus et al., 1994).

A histochemical stain commonly employed to view the various types of fibers within a muscle is reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR). It is an oxidative stain that provides a checkerboard pattern in a muscle cross section (figure 1-6), due to the number of mitochondria within each muscle fiber (Dubowitz and Sewry, 2007). Thus, since slow twitch fibers contain more mitochondria, they stain darker than the fast twitch fibers.

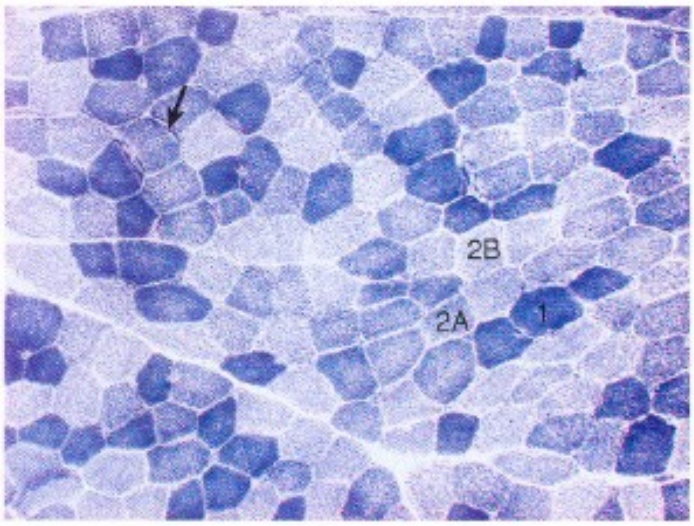


Figure 1-6: NADH stain of fiber types (Dubowitz and Sewry, 2007)

There is a relationship between the motor neurons and the muscle fibers they innervate (collectively termed a motor unit). Generally, smaller motor neurons innervate slow twitch fibers, while larger motor neurons innervate fast twitch fibers (Henneman and Olson, 1965). It has been found that the smaller units discharge earlier than the larger units (Henneman, 1957). Thus, a general rule of recruitment suggests that slow twitch motor units will activate prior to fast

twitch motor units. This is true for both static and dynamic muscle activities (Sogaard, 1995).

In practical terms, slow twitch motor units (e.g. slow twitch fibers) initially discharge. As the required force increases, the rate of discharge increases. In order to further increase tension, additional motor units (e.g. fast twitch fibers) are activated.

Derecruitment of motor units occurs in reverse order, resulting in decreasing force. In other words, the larger motor units stop firing first, progressively down to the smaller motor units (Henneman et al., 1965). As a consequence, slow twitch fibers are active more often and for longer periods of time than fast twitch fibers (Henneman and Olson, 1965).

The types of occupational muscle injury that occur due to fatigue are the result of low force prolonged/repetitive contractions. Several researchers have studied the effects of these types of injuries in trapezius muscles of workers (Hagg, 2000). It can be hypothesized that the low back muscles may experience similar types of injuries due to low force, prolonged/repetitive contractions.

These slow twitch fibers are metabolically well-suited for prolonged contractions (Henneman and Olson, 1965). However, they may become exhausted if the task is prolonged for an extended period of time with little opportunity for breaks (Sogaard, 1995). Due to this long duration activation, these fibers have been given the term “cinderella units”, since low threshold motor units are overloaded for a prolonged period of time (Sjogaard and Sogaard, 1998).

During a muscle contraction, intramuscular pressure increases. An increase in pressure can reduce blood flow to the active muscle, which reduces the energy substrates necessary for muscle contraction, affecting Ca^{2+} homeostasis (Sjogaard and Sogaard, 1998). A prolonged contraction would affect the cinderella units, as they would not obtain the required energy substrates.

CHAPTER 2 – PHYSIOLOGY OF MUSCLE EXCITATION, CONTRACTION, AND FATIGUE

2.1 Muscle Excitation and Contraction

Muscles are composed of numerous individual fibers. In order for a muscle contraction to occur, each of these fibers requires the conversion of an electrical signal into a mechanical action. Figure 2-1 provides a brief overview of the steps occurring in muscle contraction.

A motor nerve depolarizes the neuromuscular junction, resulting in an action potential to propagate along the muscle membrane, termed the sarcolemma (step 1). The action potential then travels down the transverse tubule (T-tubule), activating the dihydropyridine receptor (DHPR). The DHPR interacts with ryanodine (RYA) receptors, which triggers the release of calcium (Ca^{2+}) from the lateral sac of the sarcoplasmic reticulum (SR) (step 2).

Connected to the thin filament, actin, are two molecules: troponin and tropomyosin. Calcium binds to the troponin molecule causing it, through its attachment to tropomyosin, to move tropomyosin away from the myosin-binding site on actin. With the binding site exposed, myosin can attach to actin (step 3).

Once myosin binds to actin, Adenosine Diphosphate (ADP) and Phosphate (Pi) are released from myosin, producing cross-bridge movement (step 4). Adenosine triphosphate (ATP) attachment is then required to break this actin-myosin bond in order to prepare for a subsequent contraction. Calcium is then released from troponin, shifting tropomyosin back over the binding site, inhibiting myosin attachment (step 6). The calcium is taken back up into the SR

by the Ca^{2+} ATPase, which restores the actin and myosin proteins to their resting state, ending the contraction (step 5).

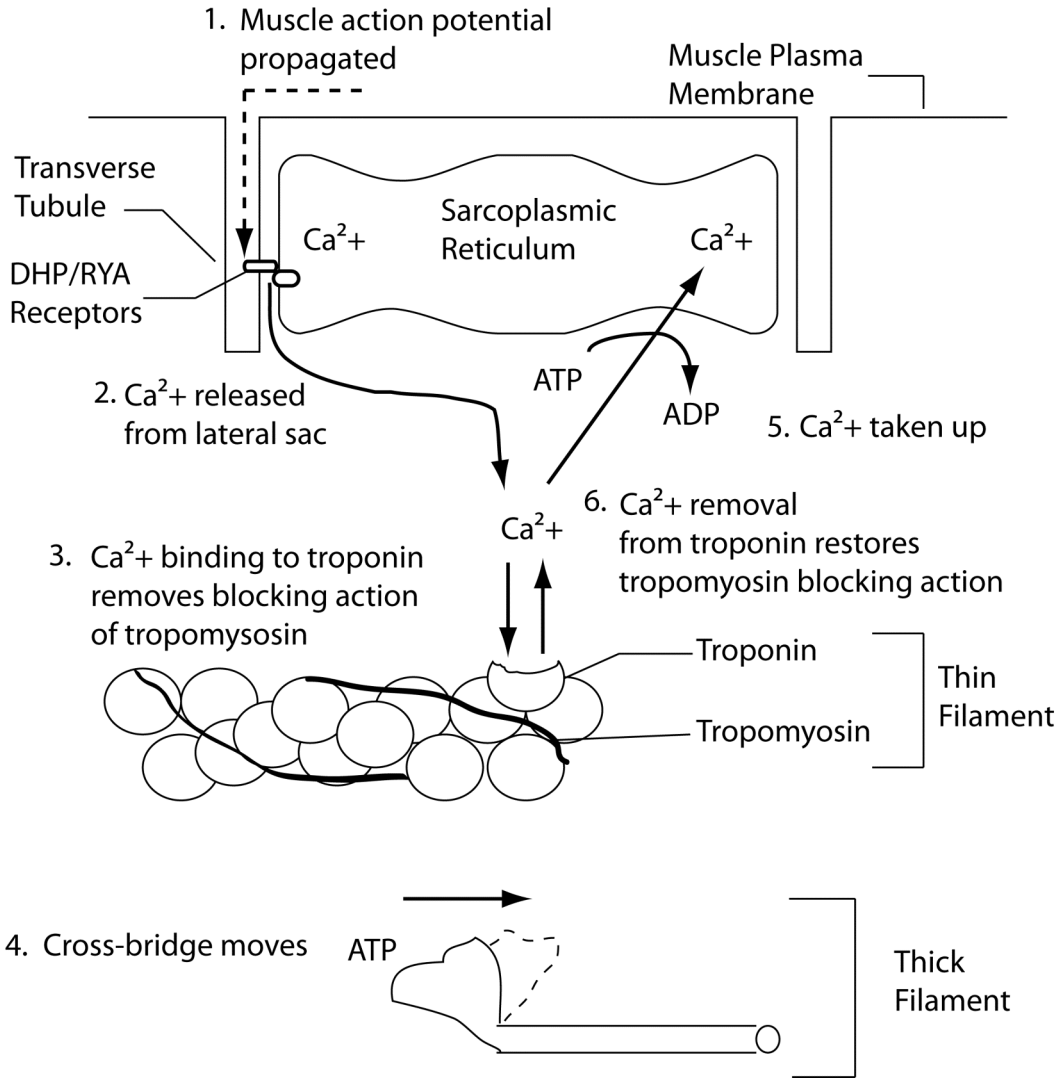


Figure 2-1: Steps for a muscle contraction (Widmaier et al., 2004).

2.2 Physiologic Basis of Muscle Fatigue

Although fatigue is a complex phenomenon in which the exact physiological mechanisms are not completely understood, it is believed that fatigue can occur at any of the steps in muscle contraction (Fitts, 2008). The cause of muscle fatigue varies based on the type of activity being performed (Baudry et al., 2009). For example, intensity and duration of the activity may

influence which step in the muscle contraction cycle fatigue occurs. The remainder of this chapter highlights the steps in muscle contraction where fatigue is likely to occur during low force prolonged contractions.

2.2.1 Sarcolemma Excitability

Ionic factors may be implicated in muscle fatigue. During an action potential along the sarcolemma, sodium (Na+) gated channels open allowing Na^+ to rush into the cell, depolarizing the muscle fiber, which provides the stimulus for a muscle contraction to occur. The Na+ gated channels are then quickly inactivated, returning the cell to a repolarized state, which stops the muscle contraction stimulus. During the Na+ inactivation, potassium (K+) is also released from the cell through K+ gated channels, speeding up the repolarization of the cell, resulting in hyperpolarization. These channels then inactivate, returning the cell to equilibrium in preparation for the next stimulus.

During fatiguing contractions, this process can lead to excessive extracellular potassium ($[K^+]_o$). Physiologically, high $[K^+]_o$ results in inexcitability of the sarcolemma, leading to reduced force output as well as endurance (Clausen, 2008). It was found that immersing fibers in high $[K^+]_o$ results in a contraction occurring only when reducing $[K^+]_o$ and then reimmersing the fiber in high $[K^+]_o$ (Hodgkin and Horowicz, 1960).

In addition to increased $[K^+]_o$, an increase in intracellular sodium ($[Na^+]_i$) can occur. In this situation, Na^+ influx will be lowered, resulting in decreased muscle action potential amplitude and eventually an inability for the action potential to propagate along the muscle fiber (Overgaard et al., 1997).

Therefore, inactivation of Na^+ channels has also been suggested to be the cause of the decreased excitability (Ruff et al., 1988).

The job of restoring the Na^+/K^+ gradients falls to the Na^+-K^+ pumps. At rest, there is a leakage of 3 Na^+ entering the cell and 2 K^+ leaving. Counteracting this flow of ions is the Na^+-K^+ pump, which transfers 3 Na^+ out and 2 K^+ into the cell. During exercise, the activity of the Na^+-K^+ pumps is elevated in response to the increased Na^+ and K^+ fluxes. Muscle fibers contain a significant amount of Na^+-K^+ pumps and these are maximally activated during intense muscle activity (Clausen et al., 1998). The trigger for the increased pump activity is partly due to an increase in $[Na^+]_i$ (Nielsen and Clausen, 1997), as well as to circulating catecholamines (Clausen, 2008).

The Na^+-K^+ pump is the limiting factor in maintaining excitability during high frequency stimulation (Harrison et al., 1997). Inhibiting the Na^+-K^+ pump by pre-incubating the muscle in ouabain, a substance that inhibits Na^+-K^+ pumps, results in decreased excitability (Nielsen et al., 2004). Conversely, adding salbutanol, adrenaline, or insulin, which are known to stimulate the Na^+-K^+ pump, results in increased force output (Overgaard et al., 1997).

During prolonged exercise, Na^+-K^+-ATPase activity may be compromised, affecting the Na^+-K^+ gradients (Fowles et al., 2002). This can lead to decreased membrane excitability, which could lead to muscle fatigue (Leppik et al., 2004). This decreased excitability can reduce the amount of Ca^{2+} release from the SR necessary to maintain force output.

2.2.2 Excitation-Contraction Coupling

Excitation-contraction (E-C) coupling encompasses depolarization of the sarcolemma (described in the previous section) and down the T-tubule, mechanisms coupling the T-tubule and SR causing a release of Ca^{2+}, as well as Ca^{2+} uptake by the SR (steps 1,2, and 5 in figure 2-1). These steps emphasize the management of Ca^{2+} release and uptake. During fatiguing contractions, it has been found that both Ca^{2+} release and reuptake is depressed (Hill et al., 2001). The reduction in Ca^{2+} release is most likely associated with the reduction in force generation (Chin et al., 1997; Hill et al., 2001), while the reuptake of Ca^{2+} may be associated with slowed relaxation of the muscle (Westerblad et al., 2000).

The T-tubule is a continuous invagination of the sarcolemma into the muscle cell between the terminal cisternae of two sarcoplasmic reticulum (figure 2-2). In response to an action potential traveling down the T-tubule, the DHPR are activated, which activate the SR Ca^{2+} release channels, RYA receptors (Westerblad et al., 2000). A strong hypothesis for reduced Ca^{2+} release and the subsequent decrease in force points to structural changes in the RYA/Ca^{2+} release channels (Lamb et al., 1995; Ortenblad et al., 2000; Westerblad et al., 2000) and not attributed to impaired activation along the T-tubule (Chin et al., 1997; Westerblad et al., 1993).

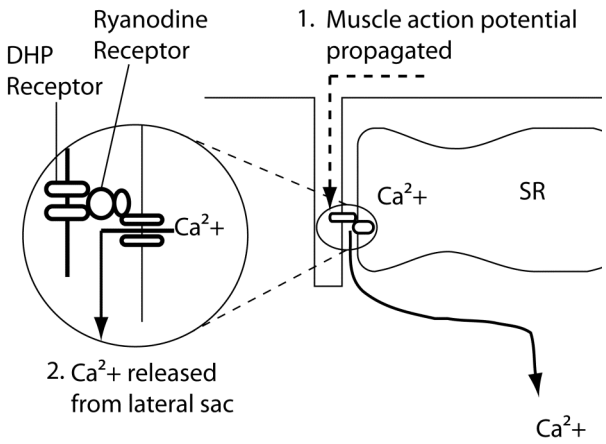


Figure 2-2: RYA receptors.

The sarcoplasmic reticulum contains a pump, the SR Ca^{2+} ATPase, which is responsible for the reuptake of Ca^{2+}. A reduced SR Ca^{2+} ATPase pump rate is believed to be the cause of increased relaxation time, which increases resting intracellular calcium ($[Ca^{2+}]_i$) (Westerblad et al., 1993). Initially, the depressed reuptake of Ca^{2+} may have the benefit of maintaining force, as the increased levels of $[Ca^{2+}]_i$ assist in the contraction of myofibrillar proteins (Leppik et al., 2004). However, this elevated $[Ca^{2+}]_i$ may contribute to further reductions in Ca^{2+} release to minimize the damaging effects of increased $[Ca^{2+}]_i$ (Chin and Allen, 1996).

Local ATP depletion may have an effect on fatigue (Steele and Duke, 2003). In very intense contractions there is a large turnover of ATP, resulting in a large production of Pi. The Pi can enter the SR and bind with Ca^{2+} stored in the SR, leading to a lowered Ca^{2+} release from the SR, reducing force generation (Allen and Westerblad, 2001). Typically, the high generation of Pi results from the breakdown of phosphocreatine (PCr), which occurs anaerobically (Westerblad et al., 2000). However, long duration activities require aerobic

metabolism, suggesting that this mechanism does not contribute to this type of fatigue (Allen and Westerblad, 2001). The metabolic effects recover relatively quickly (Chin et al., 1997) and are thus not implicated in long duration activities (Chin and Allen, 1996).

A type of fatigue termed low frequency fatigue (LFF) is of interest with respect to injury and performance, as recovery is a slow process that may take greater than 24 hours (Chin and Allen, 1996; Chin et al., 1997; Westerblad et al., 1993). LFF is most likely due to a disruption in E-C coupling (Blangsted et al., 2005; Chin et al., 1997). This type of fatigue can occur after any form of intense activity (Westerblad et al., 2000; Westerblad et al., 1993). There is evidence that LFF can occur after short maximal stimulations (Chin and Allen, 1996), as well as intermittent contractions as low as 10% MVC (Sogaard et al., 2003).

The characteristic of LFF is decreased force output at low frequency muscle activation (Blangsted et al., 2005). High frequency muscle activation such as maximal voluntary contraction (MVC) is not affected during LFF due to recruitment of high-threshold motor units in an MVC (Blangsted et al., 2005). This is because a drop in $[Ca^{2+}]_i$ affects force more at low frequency stimulation than high frequency stimulations (figure 2-3) (Westerblad et al., 2000). Thus, testing muscles in the lower force ranges will provide evidence of low frequency fatigue, rather than performing higher force contractions, such as MVCs (Blangsted et al., 2005).

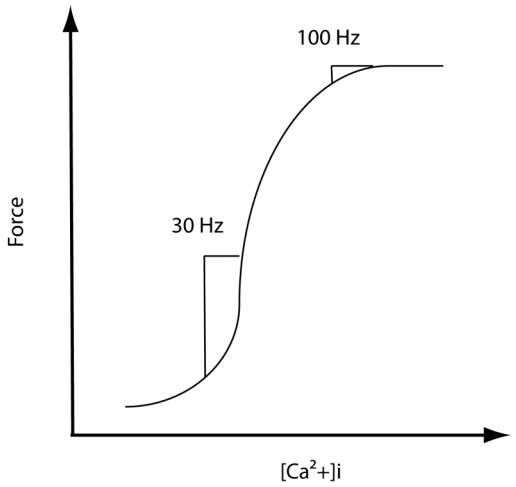


Figure 2-3: Calcium – force relationship (Westerblad et al., 2000).

2.3 Muscle Injury

Increase in $[Ca^{2+}]_i$ is cited as the trigger leading to cell membrane damage (Fredsted et al., 2007). During muscle contraction, Ca^{2+} release from the SR is necessary for force generation. However, it is believed that increased $[Ca^{2+}]_i$ results in activation of calpain, a protease, which leads to destruction of the sarcolemma (Belcastro et al., 1998). This destruction allows additional Ca^{2+} to enter the cell, further contributing to membrane damage (Allen, 2004). The Ca^{2+} activated proteases may also be responsible for damage to the SR Ca^{2+} release channel, which affects the E-C coupling (Allen, 2004).

Fortunately, the SR is responsible for the reuptake of Ca^{2+} to minimize its negative effects. Although the SR has a large Ca^{2+} storage capacity, prolonged contractions can saturate the SR storage (Gissel, 2000). The mitochondria then takes on more of the Ca^{2+} uptake and this could lead to mitochondrial damage (Gissel, 2000). The mitochondria in slow twitch “cinderella” fibers are typically damaged. This can be visualized by the NADH stain, as the fibers are unevenly

stained, suggesting there is a disruption to the myofibrils (Dubowitz and Sewry, 2007). Since the mitochondria are responsible for energy generation, damage to the mitochondria results in decreased ATP generation. This reduces the ability of the SR Ca^{2+} ATPase to uptake Ca^{2+}, further increasing $[Ca^{2+}]_i$ (Gissel, 2000).

During muscle contraction there is an influx of Na^+ due to the muscle action potential. The Na^+-K^+ pump works to extrude the Na^+ from the cell and return K^+ into the cell in order to maintain homeostasis. Muscle cells also contain a Na^+-Ca^{2+} exchanger, which goes against the Na^+-K^+ pump by bringing Na^+ into the cell and Ca^{2+} out of the cell (figure 2-4). It seems that the muscle fiber extrudes Ca^{2+} at the expense of bringing in more Na^+ because of the damaging effects Ca^{2+} can have. However, during intense and/or prolonged contractions, this exchanger may fail to work due to the increased concentration of Na^+ intracellularly, thereby further contributing to increased $[Ca^{2+}]_i$ (Everts et al., 1993).

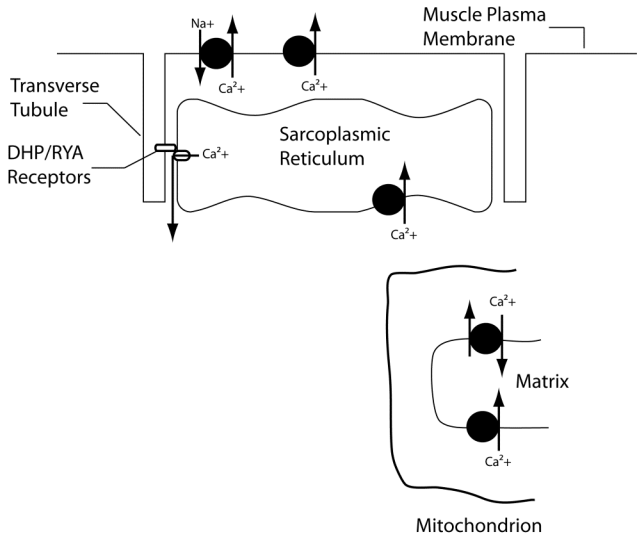


Figure 2-4: Ca^{2+} exchangers.

Extracellular Ca^{2+} also enters the cell due to membrane leakage, contributing to a further increase in $[Ca^{2+}]_i$ (Fredsted et al., 2007). During prolonged electrical stimulation, there is a longer lasting membrane leakage (Gissel and Clausen, 2003). Following prolonged electrical stimulation, during the rest period, Ca^{2+} continues to enter the cell (Gissel and Clausen, 2003).

Armstrong, et al. (1991) proposed a model of stages of muscle injury, indicating that if the contractions are stopped at a point that limits the increase in $[Ca^{2+}]_i$ then the fiber will return to normal. However, extending the duration of contractions will increase the $[Ca^{2+}]_i$, leading to muscle injury (Armstrong et al., 1991). Therefore, while Ca^{2+} is necessary for muscle contraction and the subsequent generation of force, increased Ca^{2+} due to prolonged contractions can result in injury.

CHAPTER 3 – INDUCING AND MEASURING MUSCLE FATIGUE AND INJURY

3.1 Electrical Muscle Stimulation

Electrical stimulation is often used to generate muscle contractions, particularly in animal studies. The stimulation produces a compound muscle action potential, known as an M-wave, which represents the composite of those individual muscle action potentials (Fowles et al., 2002). The purpose of measuring M-waves is to understand the behavior of muscles due to electrical stimulation.

Increasing the strength of electrical stimulation results in an increase in the number of activated motor units. This translates into an increased M-wave amplitude and area in the recording (Bigland-Ritchie, 1981). Duration of the M-wave signal represents the range of conduction velocities of the motor unit action potentials across the sarcolemma (Bigland-Ritchie, 1981). For example, the range of conduction velocities contributes to the width of the compound muscle action potential.

3.1.1 Muscle Fatigue Due To Electrical Stimulation

During continued stimulus, the M-wave amplitude has been found to initially increase, stabilize, and then decrease (Shushakov et al., 2007). The M-wave amplitude increase is due to the recruitment of activated motor units, while the decrease in amplitude is the result of reduced motor unit activity, indicating fatigue. Since the area of the M-wave signal is also determined by the number of activated muscle fibers, a decrease in M-wave area indicates fatigue (Bigland-Ritchie, 1981). Also, an indication of fatigue is an increase in the M-wave duration (Bigland-Ritchie, 1981). This is reflective of the fast twitch fibers,

containing the faster conduction velocities, being fatigued and no longer contributing to the M-wave. The remaining fibers would be slow twitch, which would have slower conduction velocities, resulting in a longer duration M-wave.

Some studies suggest that the cause of fatigue is different based on the rate of stimulation (Badier et al., 1999; Takata and Ikata, 2001). Badier et al (1999) stimulated rat tibialis and soleus muscles at 80Hz and 10Hz, and found a greater decrease in amplitude and increase in duration of the M-wave in the higher stimulation frequency condition. Takata and Ikata (2001) came to a similar conclusion when comparing 100Hz and 30Hz stimulations in rat soleus and gastrocnemius muscles, as they observed a larger decrease in M-wave amplitude with higher stimulation frequencies.

It has been found that there is a close correlation between the M-wave area and the subsequent force produced by the electrical stimulation (Clausen and Overgaard, 2000). In addition to measuring M-waves, Badier et al (1999) and Takata and Ikata (2001) measured muscle force. In both cases, higher stimulation frequencies resulted in greater force decline. Clausen et al (2004) stimulated rat extensor digitorum longus and soleus muscles intermittently (1s on 3s off) at frequencies ranging from 10 to 200 Hz and found the rate of force decline was less when stimulated intermittently than when the muscle was stimulated continuously.

A correlation has also been found between M-waves and the activity of the Na^+-K^+ pumps (Fowles et al., 2002). Different muscle types contain varying concentrations of Na^+-K^+ pumps. For example, extensor digitorum longus (EDL),

primarily a fast-twitch muscle, contains a higher percentage of Na^+-K^+ pumps than soleus, which is predominantly a slow twitch muscle. The rate of force decline at 60Hz stimulation was greater in EDL than soleus (Clausen et al., 1998). Lower stimulation frequencies (10 – 20 Hz) produced similar effects (Everts et al., 1993). It was proposed that the force decline was due to a greater influx of Na^+ per twitch in fast twitch muscles (Clausen et al., 1998).

Overgaard et al (1999) and Clausen and Overgaard (2000) performed experiments on rat soleus and EDL muscles. They blocked K^+ channels, which decreased sarcolemmal excitability, followed by chemically stimulating the Na^+-K^+ pumps, which had the effect of increasing the excitability of the sarcolemma. They found a decrease in M-wave area and force while the K^+ channels were blocked and an increase in M-wave area and force during Na^+-K^+ pump stimulation.

In summary, a decline in M-wave amplitude indicates reduced muscle membrane excitability (Behm and St-Pierre, 1997), due to an increase in $[Na^+]_i$ and $[K^+]_o$ (Takata and Ikata, 2001). The reduced membrane excitability results in a decreased release of Ca^{2+} from the SR, reducing muscle force generation (Takata and Ikata, 2001). This would then explain the correlation between decreased M-wave and reduced force output (Clausen et al., 2004). Thus, the M-wave is a good indirect measure of the excitability of the sarcolemma (Fowles et al., 2002).

3.1.2 Muscle Injury Due To Electrical Stimulation

Animal models of induced muscle fatigue leading to injury have the advantage of providing understanding of underlying physiological and cellular

mechanisms not available in human volunteer muscle fatigue studies. One method to ensure muscle injury is to perform a stretch contraction (typically referred to as eccentric contraction) of the muscle. An example set-up is to place the animal's lower limb into a motorized fixture whereby the muscle is lengthened while providing an electrical stimulus (Lieber et al., 1994). Thus, the muscle contracts but the load is causing the fibers to lengthen in eccentric contraction.

Eccentric contraction results in disruption of the sarcomeres due to mechanical damage (Lieber et al., 2002). These types of injuries typically have a greater influence on fast-twitch muscle fibers (Warren et al., 1994). While this eccentric contraction model induces injury, it does not produce the types of injuries experienced in low force prolonged/repetitive contractions. Stimulating muscles without eccentric contraction may be a better representation of the low force prolonged/repetitive injuries experienced in the workplace.

One of the effects of chronic low frequency stimulation (CFLS) is the physiological transformation of fast twitch fibers to slow twitch fibers (Antipenko et al., 1999). A stimulation frequency of 10Hz is commonly used over a period of 24 hours or more (Kraus et al., 1994). This frequency has been found to slow the contraction speed and increase fatigue resistance in fast twitch fibers (Hennig and Lomo, 1985).

There is evidence that in addition to fiber type transformation, stimulation at 10Hz can result in muscle fiber damage (Lexell et al., 1993). Increasing the stimulus frequency to 40Hz can greatly decrease the time for transformation (Kraus et al., 1994) and increase the chance for muscle injury (Gissel and

Clausen, 2000). Also, the length of time the fiber contracts determines the degradative process (Maier et al., 1986).

The results of stimulation experiments in animal models have determined that muscles with predominantly fast twitch fibers are more susceptible to injury than muscles with predominantly slow twitch muscles (Gissel and Clausen, 2000). Eventually, higher threshold (e.g. fast twitch) fibers, which are not well-suited to prolonged activity, become recruited and muscle damage may be initiated in those fibers (Gissel, 2000).

Muscles vary in terms of their natural firing frequencies. For example, the soleus muscle, with a predominant number of slow twitch fibers, has an average discharge rate between a low of approximately 8Hz and a maximum of 100Hz, while a predominantly fast twitch muscle such as EDL ranges between 30 and 200Hz (Hennig and Lomo, 1985). Stimulating fibers outside their natural firing frequencies has a greater effect on the occurrence of injury (Everts et al., 1993).

3.2 Inflammation

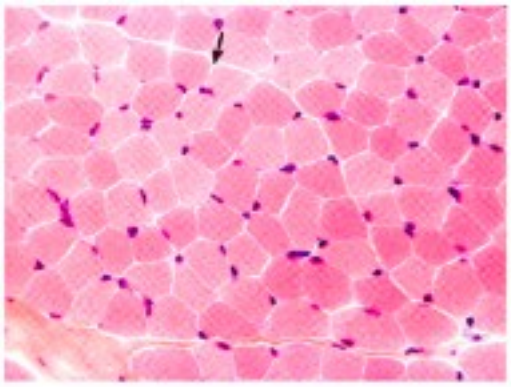
Although muscle injury can occur due to various types of trauma, including: lacerations, contusions, and strains, the response to the trauma is similar (Huard et al., 2002). Inflammation, which includes the proliferation of white blood cells, is the immediate response to that trauma. The classic signs of inflammation include: redness, swelling, heat, and pain (Butterfield et al., 2006).

The inflammatory response occurs in the event of membrane damage and entry of extracellular calcium (Stauber, 2004). However, at the onset of exercise there is an immediate increase in circulating white blood cells, due to increases in epinephrine, blood flow, and cell-signaling molecules (Butterfield et al., 2006).

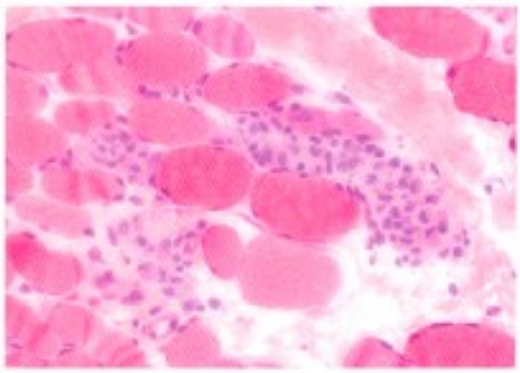
Vasodilation of local blood vessels then allows the white blood cells to enter the injured muscle tissue (Barr and Barbe, 2004).

Neutrophils are the first white blood cells to enter the muscle. They invade the muscle within 1 hour of increased muscle use and can remain elevated above baseline for up to 5 days, with their peak elevation within the first 24 hours (Smith et al., 2008; Tidball, 2005). The purpose of neutrophils is to initiate phagocytosis and remove damaged tissue (Butterfield et al., 2006). Neutrophils can also magnify the inflammatory response, leading to damage to healthy muscle tissue (Smith et al., 2008; Tidball, 2005).

Visualization of an inflammatory reaction can be performed with a Haematoxylin and Eosin (H&E) stain under a light microscope. Figure 3-1 provides images of a normal, healthy muscle and a muscle section undergoing an inflammatory response. The clusters of dark circular cells in the damaged muscle in figure 3-1B are white blood cells.



A



B

Figure 3-1: Sample H&E stains of muscle (Dubowitz and Sewry, 2007). **A)** Healthy muscle, **B)** Muscle with inflammatory response.

Macrophages are the next cells to invade muscle as part of the inflammatory response. There are 3 specific types of macrophages: ED1, ED2,

and ED3, with ED1 macrophages having the greatest known effect on the inflammatory process (Smith et al., 2008; Tidball, 2005). ED1 macrophages begin appearing within the first 3 to 24 hours, peak between 24 and 72 hours, and remain elevated until the necrotic tissue is removed, which could be approximately 1 to 3 weeks (Butterfield et al., 2006). Figure 3-2 illustrates ED1 macrophages (dark irregular shaped cells), utilizing an ED1 immunohistochemical stain under a light microscope.

Figure 3-2: Illustration of ED1 macrophages in damaged tendon (Barbe et al., 2003)

Macrophages, in addition to the role of phagocytosis (Peake et al., 2005), are responsible for releasing cytokines at the inflammation site (Ostrowski et al., 1998; Stauber and Smith, 1998). One of the cytokines, TGF-β, is responsible for stimulating collagen and an increase in fibroblasts, which can inhibit the muscle repair process in chronic injury (Smith et al., 2007). This connective tissue inhibits muscle fiber regeneration (Hurme et al., 1991), which can decrease tissue load tolerance, allowing lower levels of exertion to contribute to further tissue damage (Barr and Barbe, 2004).

Although eccentric contractions produce a marked inflammatory response, there is evidence that chronic repetitive movements can result in inflammation. Barbe et al, (2003) induced MSDs in rats, as evidenced by macrophages, by

having them repetitively reach for food for 2 hours/day, 3 days/week, for 3 to 8 weeks. Chronic inflammation can be caused by repeated injury (Barbe et al., 2003). It has been found that MSDs are the result of overuse associated with inflammation (Carp et al., 2007). The repeated bouts of inflammation due to muscle overuse can affect muscle regeneration (Barr and Barbe, 2004).

3.3 Muscle Damage

Muscle damage is produced in a small portion of fibers, even in long duration running events (Overgaard et al., 2002). These small number of fibers can cause considerable pain since the nociceptors sense cellular damage (Armstrong, 1986). Muscle pain is a good indicator of the extent of damage as it peaks 1-3 days after the event and is still present 7 days later (Armstrong, 1986).

There is evidence to suggest that slow twitch fibers (cinderella units) are damaged in low force, prolonged/repetitive contractions. Muscle biopsy studies performed on trapezius muscles of female cleaners indicated damage to slow twitch muscle fibers compared to controls (Kadi et al., 1998; Larsson et al., 2000; Larsson et al., 2004). This was determined by using the NADH-TR histochemical stain. Fibers give the appearance of being moth-eaten due to myofibrillary disruption within the cells (figure 3-3). This moth eaten fiber appearance indicates that there is disruption in the oxidative metabolism possibly due to ischemia, suggesting damage to the mitochondria (Larsson et al., 2004).

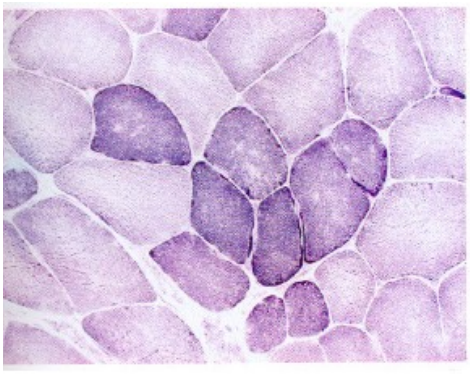


Figure 3-3: Example moth eaten fibers (Dubowitz and Sewry, 2007). Dark stained slow twitch fibers have portions of the cell where it is not continuous.

Structural breakdown of the muscle fiber can occur in injury. Desmin is the primary intermediate filament (Carlsson and Thornell, 2001) responsible for providing mechanical stability to the muscle fiber (Lieber et al., 2002). Desmin links adjacent z disks (Lieber et al., 2002), which may help it serve as a transmitter of tension between adjacent sarcomeres (Li et al., 1997) (figure 3-4).

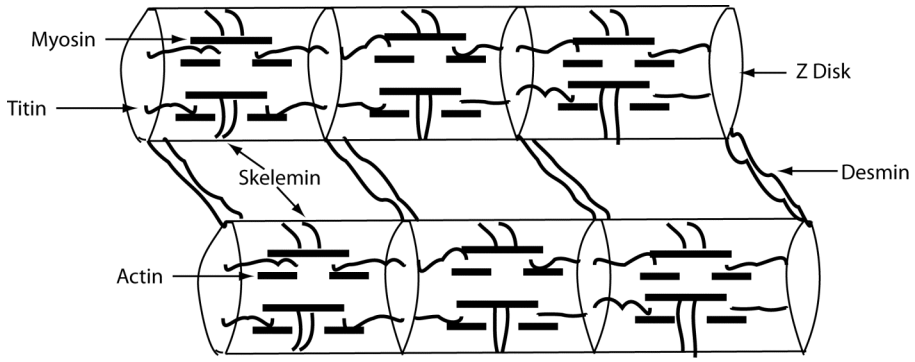


Figure 3-4: Structure of muscle cytoskeleton, highlighting the connection of the Z disks with desmin.

Applying an immunohistochemistry stain for desmin allows for the visualization of damage to this intermediate filament. In this case a lack of desmin stain indicates damage. Vater et al, (1992) induced muscle degeneration due to influx of Ca^{2+} by inoculating rats with snake venom. They found absence of desmin in some fibers within 3 hours and regeneration with the presence of

desmin 2 days after the venom injection (Vater et al., 1992). Desmin staining is lost at an early stage of necrosis and is present early in regeneration (Helliwell, 1988).

3.4 Muscle Regeneration

The purpose of inflammation is to repair damaged muscle tissue (Barbe and Barr, 2006). In fact the presence of macrophages appear to be the trigger for muscle regeneration (Carlson and Faulkner, 1983). Without macrophage infiltration muscle regeneration does not occur and the fiber remains in a degenerative state (Carlson and Faulkner, 1983).

Macrophage activity triggers satellite cells into regenerating muscle fibers (Hurme et al., 1991). Satellite cells are stem cells located beneath the basal lamina (Allbrook, 1981). Upon activation satellite cells form myoblasts, which fuse into myotubes and then muscle fibers (Carlson and Faulkner, 1983). The process of forming new fibers occurs within 2 weeks, while just after 4 days post event, myotubes are formed (Bornemann and Schmalbruch, 1992). These new fibers are added onto the existing fibers (Allbrook, 1981). Although the sarcolemma becomes damaged in muscle fiber injury, the basal lamina has increased resistance to injury, protecting the satellite cells (Carlson and Faulkner, 1983). Mature fibers, characterized by peripherally located rather than centrally located nuclei (Carlson and Faulkner, 1983), develop after 3 weeks (Bornemann and Schmalbruch, 1992; Hurme et al., 1991).

Immunohistochemical staining for desmin, a major component of z-discs (Putman et al., 1999), and vimentin, linking z-discs in regenerating muscle (Vater et al., 1994) provides useful information regarding the status of muscle fiber

regeneration (Bornemann and Schmalbruch, 1992). Vimentin appears to act in regenerating fibers, peaking at 2 – 3 days (Vater et al., 1994). As vimentin activity declines desmin expression increases (Vater et al., 1994).

CHAPTER 4 – *IN VIVO* ELECTRICAL STIMULATION AND ELECTROMYOGRAPHIC RECORDING OF THE MEDIAL LONGISSIMUS RAT MUSCLE

4.1 Introduction and Rationale

Electrical stimulation is a commonly used technique to study muscle properties. A maximal electrical stimulus produces a compound muscle action potential (M-wave) that is closely correlated to the membrane potential and the subsequent force produced by electrical stimulation (Clausen and Overgaard, 2000), suggesting that the M-wave provides an indirect measure of the excitability of the sarcolemma (Fowles et al., 2002). Common methods to measure M-waves in animal models include *in vitro* experiments to study the ionic effects of the stimulation (Badier et al., 1999; Overgaard et al., 1999) and *in situ* experiments to measure the force as a result of the stimulation (Takata and Ikata, 2001). Unlike *in vivo* experiments, *in situ* models alter the natural behavior and environment of the muscle. Also, these types of experiments have been relatively short in duration, ranging from 2 to 60 minutes in length (Badier et al., 1999; Clausen et al., 2004; Karelis et al., 2002; Takata and Ikata, 2001).

While there have been several published studies utilizing *in vivo* electrical muscle stimulation, M-waves were not recorded during the *in vivo* stimulation (Egginton and Hudlicka, 2000; Eken and Gundersen, 1988; Hudlicka et al., 1994; Takahashi and Hood, 1993). In these experiments stimulating electrodes were surgically sutured to the muscles near or in contact with the nerve and the tissues were harvested at the end of the stimulation period to observe changes in contractile activity due to the stimulation. In contrast, recording electrodes also have been utilized *in vivo* to record voluntary muscle contractions (Whelan,

2003). In *in vivo* animal models M-wave recordings made with concurrent use of indwelling stimulation and recording electrodes has not been performed. This approach would provide the advantage of studying M-waves in the animal's natural environment, allowing for a potentially longer duration assessment of electrical responses.

The hind limb is the most common location used for electrical stimulation experiments in animals. In particular, the muscles of interest are typically the soleus and extensor digitorum longus (EDL) due to disparate morphology and relative accessibility (Eken and Gundersen, 1988). An alternative location to be considered for recording M-waves is the low back region. Specifically, the medial longissimus muscle (figure 4-1) is an appropriate rodent back muscle to be utilized in M-wave measurements due to its length and orientation. It is a spindle shaped muscle, spanning the entire length of the lumbar spine, originating in the L1 or L2 spinous processes and inserting in the caudal lumbar spinous processes (Brink and Pfaff, 1980). A benefit of a longer muscle is that it allows for recording electrodes and stimulating electrodes to be separated sufficiently in distance such that the stimulus artifact does not overlap the signal of interest.

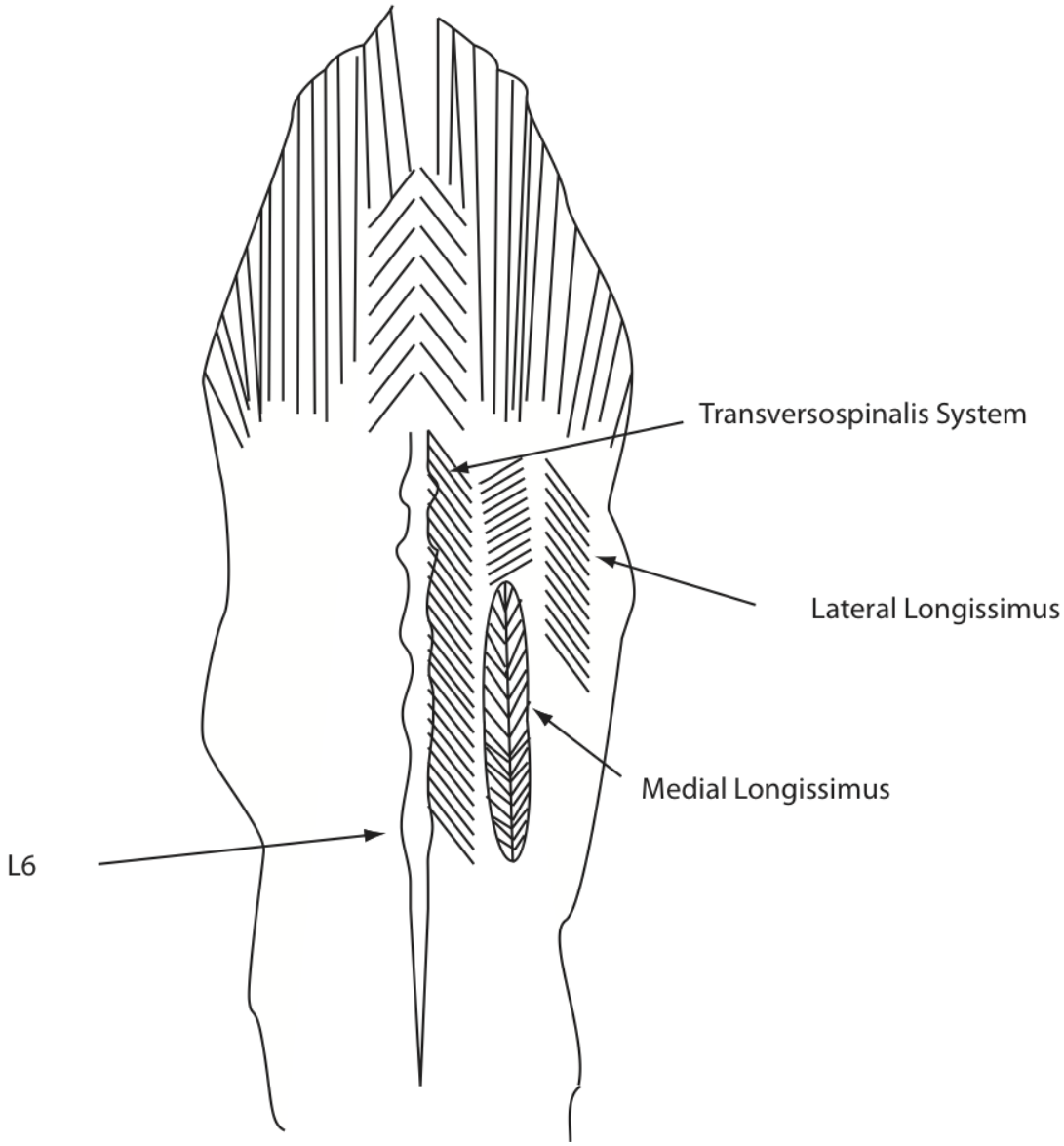


Figure 4-1: Schematic representation of the rat back anatomy. L6 is lumbar vertebrae 6.

Back muscles of rats are also of interest due to similarities with human back muscles. The function of back musculature in both species is to generate large forces and mobilize the spine (Schilling et al., 2005). These functions require different types of muscle fibers. For example, predominantly fast twitch muscle fibers are used to generate large forces, while slow twitch muscle fibers are required for spine stability. Investigating the muscles responsible for these

activities found similar fiber type distributions between rats and humans (Schilling et al., 2005). Unknown though is electrical properties in back muscles in both species. In humans, this may be due to the difficulty in measuring intracellular muscle properties or biopsying the muscle due to anatomical location.

The similarities between back muscles in humans and rats and the lack of knowledge of their electrical properties prompt the need to consider and develop methods to study back muscle in rat models to better understand common ailments associated with low back muscles in humans. Low back pain is consistently the most frequently injured body part at work (Silverstein et al., 2002) experienced by approximately 70-85% of the population at least once in their lifetime (Andersson, 1999). While the cause of low back pain may have various origins, research has shown that low back pain patients exhibit greater muscle fatigue than healthy people, as measured by surface electromyography (EMG) (Roy and Oddsson, 1998).

Fatigue, defined as an inability to maintain force, is a commonly measured variable in electrical stimulation experiments (Allen, 2004; Badier et al., 1999; Clausen et al., 2004). Results from limb muscles indicate reduced membrane excitability, resulting in fatigue (Behm and St-Pierre, 1997). It has been found in both human and animal models that during fatiguing stimulation, increased extracellular potassium ions and increased intracellular sodium ions correlate to a decrease in M-wave amplitude and area, as well as an increase in its duration (Bigland-Ritchie, 1981; Overgaard et al., 1999).

4.2 Methods

4.2.1 Electrode Fabrication

All the electrodes were made using Teflon®-insulated single stranded stainless steel wire with an outside diameter of 200μm (A-M Systems, Inc., Carlsborg, WA). The fabrication of the electrodes were based on studies in mice by Pearson et al (2005), with the exception of using a larger diameter wire for the current rat studies. For the recording electrodes two pieces of wire were lightly twisted together and a knot was tied several centimeters from one end of the pair, which was designated the proximal end (figure 4-2a). The opposite end was termed the distal end. A few millimeters distal to the knot, approximately 1mm of the Teflon insulation was removed from one wire and approximately 1mm distally from this point, 1mm of the Teflon coating was removed from the second wire (Pearson et al., 2005). These bared pieces of wire served as the recording portions of the electrode. The distal ends of the wires were inserted into a stainless steel surgical needle (MS 192-16, Miltex Instrument Company, Lake Success, NY).

The stimulating electrode was configured similarly to the recording electrode, other than being intertwined. It also contained a knot tied several centimeters from one end. A few millimeters distal to the knot, approximately 1mm of the Teflon insulation was removed, which served as the stimulating portion of the electrode. The distal end of the wire was inserted into a stainless steel surgical needle (MS 192-16, Miltex Instrument Company, Lake Success, NY). This configuration was repeated for a second stimulating electrode.

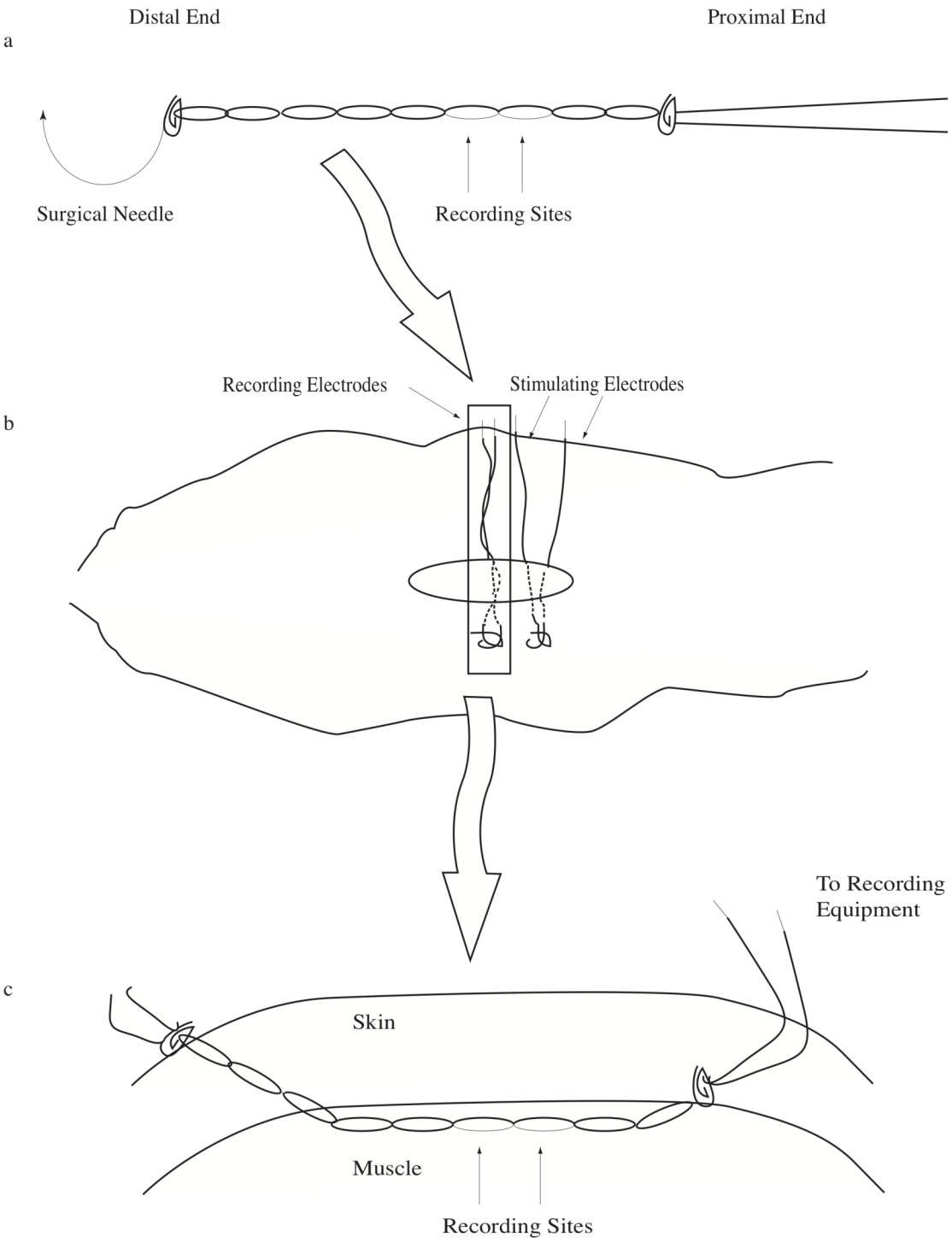


Figure 4-2: Schematic representation of electrode configuration. **a)** A pair of intertwined recording electrodes attached to a surgical needle. **b)** Experimental set-up of the electrodes surgically inserted into the medial longissimus muscle of a rat. **c)** Example of insertion of a pair of intertwined recording electrodes into the medial longissimus muscle and exiting the skin.

4.2.2 Electrode Implantation

Male Sprague Dawley rats (400 – 450g) were anesthetized with an intraperitoneal (IP) administration of ketamine (80 mg/kg) and xylazine (8-10 mg/kg). The animals were allowed to breathe independently and body temperature was kept at 36°C-38°C with a thermostat regulated thermal blanket. During the procedure ketamine (20-40 mg/kg) and xylazine (2-6 mg/kg) were administered IP, as maintenance anesthesia. Depth of anesthesia was monitored using paw pinch and palpebral reflexes.

A small incision, approximately 20mm in length was made in the skin, midline, overlying the lumbar portion of the spinal column. The surgical needle with the fine wire-stimulating electrode attached was inserted into the medial longissimus muscle left of midline through the incision at the level of L5. Anatomically in the rat, L5 is located at the level of the top of the iliac crest, which is easily palpated. A second stimulating wire electrode was inserted 17mm rostral to the first stimulating electrode. Also, a pair of intertwined recording electrodes was inserted 10mm rostral to the second stimulating electrode into the medial longissimus muscle. Thus, each rat contained a pair of intertwined recording electrodes and 2 separate stimulating electrodes (figure 4-2b).

The stimulating and recording electrodes were inserted through the medial longissimus until the pre-made knots in the wires were firmly positioned against the surface of the muscle (Pearson et al., 2005). The electrodes were then inserted into the underside of the skin, pulled through, and a knot was tied for each electrode and secured against the exterior surface of the skin (figure 4-2c). This was a modification to the Pearson et al (2005) technique, as they tied the

knot exterior to the muscle surface. The proximal end of the wire was cut approximately 3-4 cm from the knot that was originally placed against the muscle surface. Approximately 1mm of the end of the wire was bared and this termination served as the connection point to the stimulating equipment. The skin was then sutured leaving the proximal electrode ends exterior to the skin surface.

4.2.3 Electromyography Recording

A silver-silver chloride surface electrode was secured to the rat rear paw to ground the recording. The recording electrode pair was input to a customized differential AC amplifier of 110 db common mode rejection ratio, with a gain capability of up to 60,000, and a 10-2,500 Hz analog band-pass filter (Bortec Biomedical, Ltd., Calgary, AB, Canada). The channel was digitized at a rate of 5 kHz using an analog-to-digital conversion card (PCI 6036E, National Instruments Corporation, Austin, TX). Signals from the stimulator were digitized concurrently with the amplified EMG signal (gain 1 K), recorded using customized data collection software (LabView 7.1, National Instruments Corporation, Austin, TX), and stored on a computer for off-line analysis.

4.2.4 Stimulation Protocol

Uniphasic pulse stimulus (A-M Systems, Inc., Carlsborg, WA) of 3 V, 30 Hz frequency with pulse duration of 0.2 ms was applied directly to the muscle. During a pilot study it was found that 3 V produced movement and vibration in the rat-tail without movement of the rump. The 30 Hz frequency, which was used in studies by Clausen (2000) and Overgaard (1999), was selected to produce fatigue and allow for muscle activity to persist over several hours. The pulse

duration of 0.2 ms, utilized in other studies (Badier et al., 1999; Clausen et al., 2004), was found to provide distinguishable M-wave phases.

The animals were separated into 4 groups, encompassing different patterns of stimulation and rest duration (table 4-1). The parameters that were varied were duty cycle and cycle time. Cycle time refers to the total time a specific amount of stimulation and rest were applied before repeating that pattern. Duty cycle is calculated as the percentage of stimulation time divided by cycle time. For example, a duty cycle of 0.25 and cycle time of 20 seconds means that stimulation will be applied for 5 seconds followed by 15 seconds of rest. Throughout the stimulation procedures, electrical muscle activity was recorded.

Table 4-1: Electrical muscle stimulation protocol work/rest ratios

		Cycle Time	
		20 seconds	**180 seconds**
Duty Cycle	**0.25 (25%)**	5 seconds stimulation 15 seconds rest n=8	45 seconds stimulation 135 seconds rest n=6
	0.75 (75%)	15 seconds stimulation 5 seconds rest n=8	135 seconds stimulation 45 seconds rest n=6

4.2.5 Artifact and M-wave Analysis

During the initial 1-second epoch in a given cycle, 30 stimulations were recorded. Beginning at the fourth stimulation of that 1-second epoch, 10 samples were used for analysis (figure 4-3a). In each of the cycles evaluated the first epoch was used for analysis, as described above. All analyses were performed using Biopac Pro pre-packaged software (Biopac Systems, Inc., Goleta, CA).

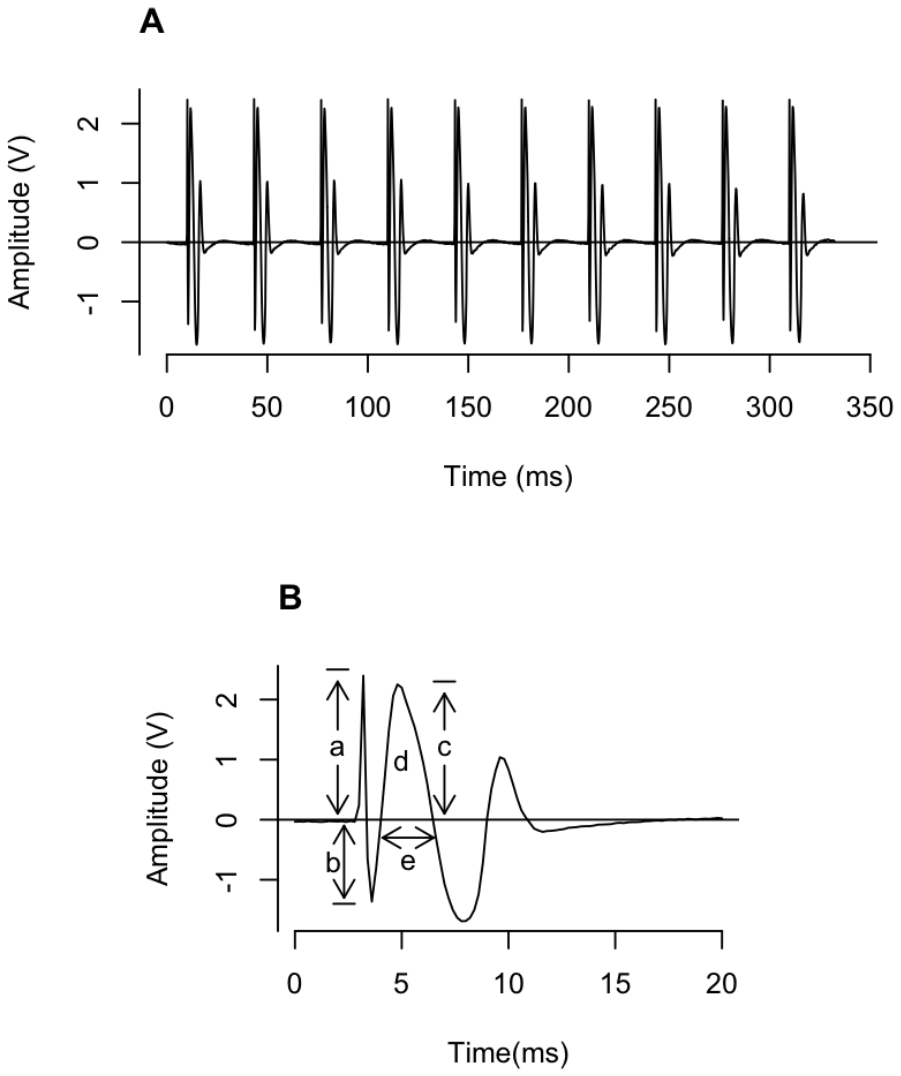


Figure 4-3: Sample M-wave with accompanying stimulus artifact. **A)** Train of 10 representative M-waves with stimulus artifacts obtained from a 1 second epoch. **B)** Single M-wave and stimulus artifact obtained from the train of 10 in A. Measurements include: a. stimulus artifact positive peak amplitude, b. stimulus artifact negative peak amplitude, c. M-wave maximum amplitude, d. M-wave area under the curve, and e. M-wave duration.

The initial positive and negative peaks in a recording were the stimulus artifacts. The amplitude of the stimulus artifact was analyzed to determine stability of the stimulus and recording electrodes. Specifically, amplitude was separated into maximum positive and negative amplitude. The maximum

positive amplitude was measured from baseline to maximum positive peak (figure 4-3b). Conversely, maximum negative amplitude was measured from baseline to maximum negative peak.

The portion of the M-wave used in this analysis was contained within the first peak following the stimulus artifact. Specifically, the analysis began at the first zero crossing following the stimulus artifact and concluded at the next zero crossing (Preston and Shapiro, 1998). A second smaller peak appeared in various experiments and based on pilot studies, it was determined that this second peak was due to additional muscles being recruited by the stimulation. Thus, for consistent analysis, only the first M-wave peak was analyzed (Preston and Shapiro, 1998). Although the first peak changed over time, it remained throughout the experiment, allowing for quantitative analysis. Within this portion of the M-wave, peak amplitude, area under the curve, and duration were analyzed (figure 4-3b). Amplitude was measured from baseline to peak, while area and duration were bound by the zero crossings.

4.3 Establishing a methodology for recording M-waves from *in vivo* electrical stimulation of the rat medial longissimus muscle

4.3.1 Specific Aim I

With the understanding that stimulating and recording from the rat medial longissimus muscle *in vivo* was unique, a methodology was developed to perform these measurements. A group of 8 rats from the 75% duty cycle and 20s cycle time group was evaluated for this methodology. The purpose of this study was to record M-waves *in vivo* in the medial longissimus muscle of the rat with the goal to determine the viability of indwelling and recording electrodes in

the same muscle. The analysis consisted of determining the stability of the electrodes by measuring the stimulus artifact and the M-wave signals over time.

4.3.2 Statistical Analysis

There were seven cycles analyzed throughout the experiment: the initial cycle, 0.05 hours, 0.5 hours, 1 hour, 2 hours, 4 hours, and 6 hours, providing a comprehensive assessment of long duration muscle activity invoked by electrical stimulation. At each of the seven cycles, for 10 waveforms the mean and standard error was calculated for stimulus artifact and M-wave measurements from 8 rats. Specifically, the stimulus artifact maximum positive and negative amplitudes were averaged, as well as the M-wave amplitude, area, and duration. The nonparametric Friedman test was performed to determine any significant differences between the seven cycles for each of the stimulus artifact and M-wave data. Where applicable, the Wilcoxin test was performed to identify which point(s) showed the greatest changes. These nonparametric tests were used due to non-normal distribution of M-wave measurements. An alpha level of $p<0.05$ was considered significant for these analyses. All statistical procedures were performed in PASW Statistics v18.1 (SPSS, Inc Chicago, IL).

4.3.3 Results

A total of eight rats were included in this study and all eight survived the six-hour experiment. Six of the eight rats survived three days after the experiment before being sacrificed, while two of the rats expired within three days, due to complications with the anesthesia. The six surviving rats recovered normally and showed no indications of distress due to the electrodes, which remained in the rats for the three days leading up to sacrifice.

The surgery to insert the electrodes took an average of 30 minutes per rat. The medial longissimus muscle was readily located once the skin was incised. This location was verified upon 30 Hz stimulation, as the tail of each rat vibrated, indicating that the medial longissimus muscle was being stimulated (Brink and Pfaff, 1980). Also the location of the recording electrodes was confirmed to be in the medial longissimus muscle, as typical M-waves with corresponding stimulus artifact were produced (figure 4-3). Since the rats were anesthetized during the experiment, there was minimal movement of the electrodes. Upon sacrifice, the electrodes were visually found to be secure in the muscle, with the knots in their original positions.

4.3.3.1 Stimulus Artifact

Electrical stimulation was administered at a constant voltage and was not subject to biological variability, thus the artifact recorded with the physiological signal provided a means to assess integrity of recording for the 6-hour duration. Figure 4-4 shows the measurements over several cycles from a representative experiment where the beginning positive and negative peak of each stimulus was easily identified throughout the experiment. The stimulus artifact data was contained within the initial positive and negative peaks, ending with the zero crossing following the negative peak, which was a consistently measurable point. The sample signals in figure 4-4 were randomly selected from each of the 7 cycles evaluated.

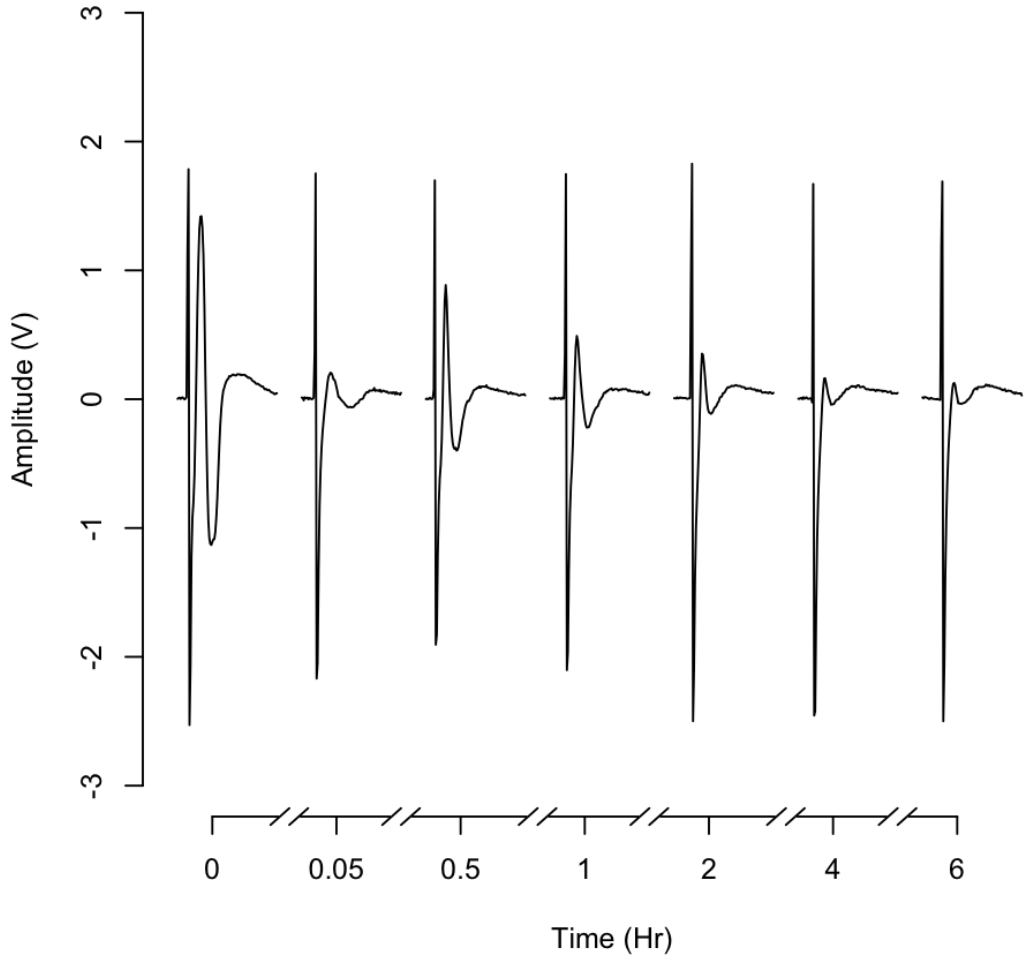


Figure 4-4: Samples of M-waves taken at baseline and 6 cycles: 0.05, 0.5, 1, 2, 4, and 6 hours during the experiment for a representative rat.

The stimulus artifact positive amplitude in figure 4-4 shows little variation, while the negative amplitude was more variable. This representative sample was consistent with the average positive and negative stimulus artifact amplitudes (table 4-2). From the 3-minute cycle until 6 hours, the positive peak amplitude varied between 97.4% and 99.4% of the first cycle. The negative peak amplitude was slightly more variable, with a range between 99.5% and 104.1% compared

to the first cycle. The results showed that there was no significant effect at any of the cycles on maximum peak ($p>0.29$) or minimum peak of the stimulus artifact ($p>0.97$).

Table 4-2: Descriptive statistics for stimulus artifact positive and negative peak amplitude (V) for 7 time periods.

Amplitude	N	Range	Minimum	Maximum	Mean	Std. Error	Percent of 0 Hr
Max 0Hr	8	.20	2.31	2.50	2.40	.023	100.00
Max 0.05Hr	8	.19	2.28	2.47	2.37	.022	98.64
Max 0.5Hr	8	.51	2.01	2.52	2.35	.054	97.66
Max 1Hr	8	.25	2.26	2.51	2.37	.028	98.64
Max 2Hr	8	.18	2.31	2.50	2.39	.024	99.38
Max 4Hr	8	.19	2.31	2.50	2.39	.024	99.38
Max 6Hr	8	.23	2.27	2.50	2.38	.027	99.09
Min 0Hr	8	.43	-1.77	-1.34	-1.55	.056	100.00
Min 0.05Hr	8	.55	-1.86	-1.31	-1.54	.070	99.46
Min 0.5Hr	8	.42	-1.79	-1.37	-1.58	.046	103.24
Min 1Hr	8	.44	-1.78	-1.34	-1.53	.045	99.64
Min 2Hr	8	.46	-1.75	-1.30	-1.59	.059	104.05
Min 4Hr	8	.40	-1.69	-1.30	-1.53	.050	99.84
Min 6Hr	8	.56	-1.68	-1.12	-1.52	.068	99.55

4.3.3.2 M-wave

A representative M-wave was evident in the signal, which followed the stimulus artifact (figure 4-4) for several time points from a typical recording session. There was a large decrease in amplitude from the initial to the 3-minute cycle, suggesting fatigue due to the stimulation. There appeared to be recovery of the signal at 30 minutes before the M-wave decreased as the experiment progressed towards 6 hours. This recovery at 30 minutes followed by progressive decline was observed in 4 rats, while the other 4 rats showed a progressive decline in the signal over time with no initial recovery. Overall, the

M-waves from the 3-minute cycle until the final measurement at 6 hours were smaller than the initial cycle.

At 3 minutes of stimulation, the average M-wave amplitude decreased to approximately 34% of the initial cycle (figure 4-5). The amplitude then recovered at 30 minutes to 43% of the initial cycle. From 2 hours until the end of the experiment, the amplitude was maintained between 35% and 37% of the initial cycle. The M-waves at 3 minutes and 30 minutes represent the largest range of amplitude fluctuation. The Friedman test found a significant effect of cycle on maximum amplitude of the M-wave ($p<0.01$). Each of the cycles was significantly different from the initial cycle ($p<0.02$). Also, the amplitude difference between 1 and 2 hours proved to be significant ($p<0.02$). There were no differences between the amplitudes of the remaining cycles.

Similar to amplitude, M-wave area measurements over time produced a significant effect ($p<0.03$). At 3 minutes of stimulation the area decreased to approximately 48% of the original signal and then remained within the range of approximately 46% and 57% of the initial signal (figure 4-5). Each cycle was significantly different from the initial measurement ($p<0.05$), except at 1 hour ($p>0.05$). Also, the area at 2 hours was significantly different than the area at 1 hour ($p<0.05$), while there were no significant differences between the remaining comparisons, based on the Wilcoxin test.

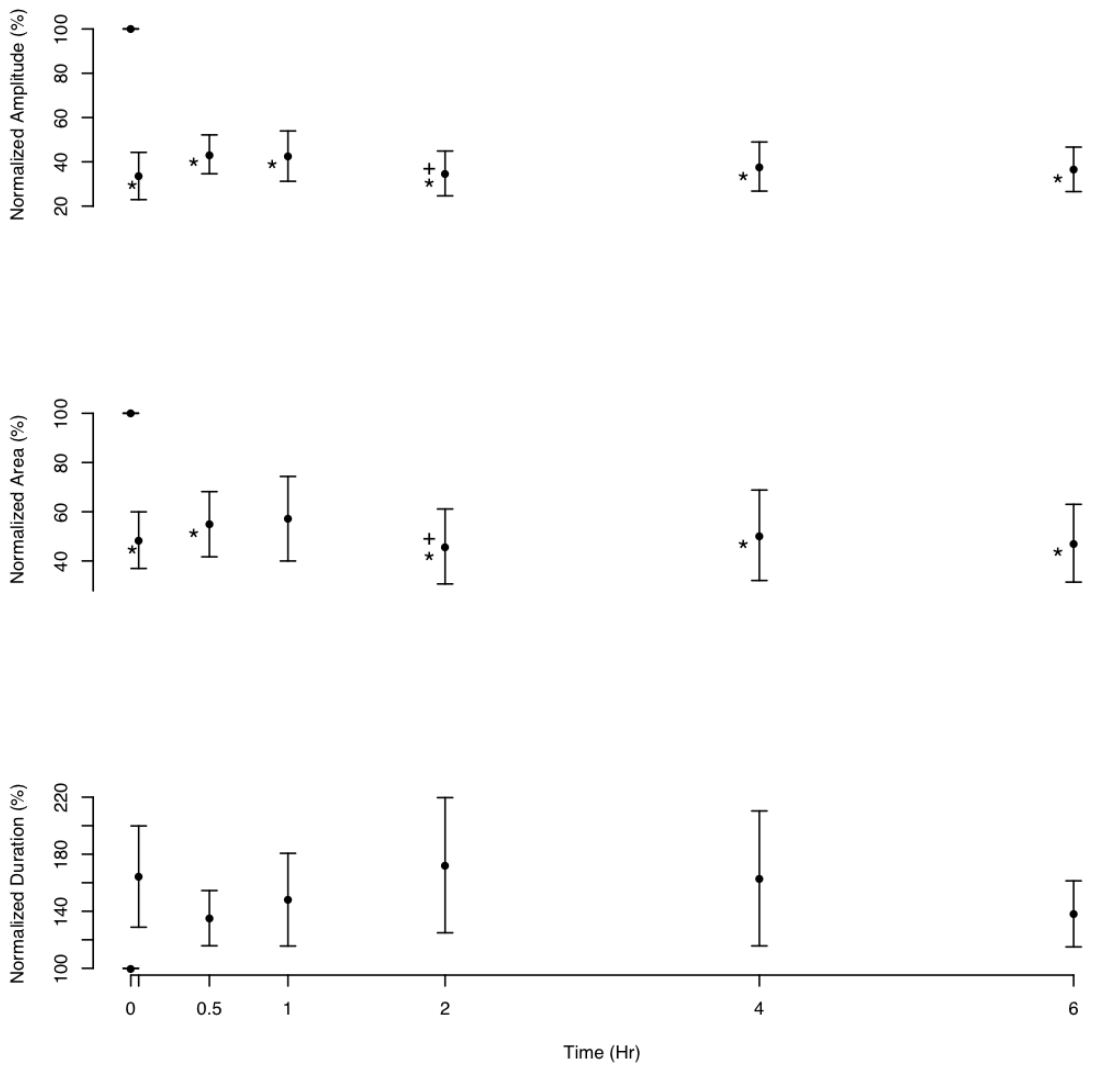


Figure 4-5: M-wave values over 7 cycles normalized to the first M-wave. a) Average M-wave maximum amplitude. b) Average M-wave area under the curve. c) Average M-wave duration. Values are means +/- SE (n=8). * Significantly different from the initial value ($p < 0.05$). + Significantly different from the previous cycle ($p < 0.05$).

Although the average duration of the M-wave increased compared to the original M-wave duration, the increase for each cycle was not significant ($p>0.50$). The duration of the M-wave increased at 3 minutes to approximately 164% of the initial signal and dropped to approximately 135% at 30 minutes (figure 4-5). There was an increase of the duration until 2 hours, before gradually

decreasing again. Duration at each cycle was greater than the initial measurement.

4.3.4 Discussion

The present study has provided evidence that muscle fatigue occurs with an *in vivo* model in the rat. This *in vivo* preparation included surgically implanted stimulating and recording electrodes in the rat medial longissimus muscle. The M-wave amplitude and area decreased significantly at 3 minutes and continued to be significantly different from the initial cycle up to 6 hours of stimulation. Duration of the M-wave signal, while not significant, was increased compared to the initial duration measurement.

The fatigue effect in the present *in vivo* experiment is similar to electrical stimulation *in vitro* and *in situ*, measured as decreased amplitude and area, and increased duration of the M-wave. For example, Karelis et al (2002) stimulated the plantaris muscle in rats at 50Hz for a 2.7s cycle while varying the length of the muscle *in situ*. They found a significant difference in M-wave amplitude and duration at 1 minute compared to the initial value. By 5 minutes of stimulation, all measurements settled at their new baseline for the remaining 55 minutes, and the M-wave amplitude, area, and duration were significantly different compared to the initial time point.

Stimulation frequency does not alter the general trend of the decay in the M-wave signal, only the magnitude. For example, Takata and Ikata (2001) electrically stimulated rat gastrocnemius and soleus muscles at 30Hz and 100Hz for 20 minutes. At 30Hz stimulation frequency, M-wave amplitude decreased quickly (by 2 minutes) and was subsequently maintained for 20 minutes.

Stimulation at 100Hz caused a larger decrease in amplitude. The present study utilized a stimulation frequency of 30Hz and it provided similar magnitude changes to the 30Hz stimulation frequencies observed in Takata and Ikata (2001).

The medial longissimus is a suitable muscle for studies of fatigue in M-wave experiments. In addition to the fatigue of back musculature being consistent with hind limb studies, the stimulus artifact signal was analyzed and consistent with prior literature. Electrical stimulation of a unipolar pulse produces an artifact that is recorded as a bipolar output due to the potential difference observed between the two recording electrodes (Geddes, 1972). This bipolar artifact was observed at the beginning of each stimulus elicited over the 6-hour electrical fatigue paradigm (figure 4). One proposed advantage of utilizing the medial longissimus is that it contains longer muscle fibers than other paraspinal muscles, which decreases the overlap between the stimulus artifact and the M-wave signal, due to the separation of stimulating and recording electrodes. The artifact was not significantly different throughout the entire 6 hours for each rat, suggesting that there was sufficient separation between the artifact and M-wave signals. This constant artifact output also represents a stable stimulating and recording signal over time.

This experiment corresponded to previous studies indicating that electrical stimulation produced fatigue within the first few minutes. There was an indication however, that a prolonged period of time may eventually lead to changes in M-wave parameters. Previous experiments (Bigland-Ritchie, 1981; Overgaard et

al., 1999) and this study have demonstrated an increase in M-wave duration initially. In the analysis of the M-wave duration, beginning at 4 hours and continuing to 6 hours, the duration decreased but not significantly. Typically, duration is related to conduction velocity of the action potential. However, it is unlikely that conduction velocity would increase at this point in the experiment, which would have the effect of decreasing duration. The decrease in duration may have been due to a further decrement in the M-wave signal, in which it reached the zero point sooner. Prolonging the experiment may eventually have resulted in a reduction in area due to this quicker return to zero.

The length of this study has provided an indication that additional modification may occur in muscle with increased duration of activity. Thus, while short-term experiments have shown that the M-wave fatigue measurements remain relatively constant, prolonged stimulation may induce additional changes in the parameters, suggesting alterations in the electrophysiological activity.

4.4 Muscle Fatigue Due to Electrical Stimulation with Varied Duty Cycles and Cycle Times – 1 Day Experiments

4.4.1 Specific Aim II

The previous study (section 4.3) provided support for the electrical stimulation and recording of M-waves within the medial longissimus of the rat. This was accomplished utilizing a single group of rats exposed to a stimulation protocol with a specific duty cycle and cycle time. The purpose of this study was to expand the number of stimulation protocols to determine the effect of various duty cycles and cycle times on muscle fatigue, as measured by M-wave amplitude, area, and duration.

4.4.2 Statistical Analysis

The analyses were performed on 28 Male Sprague-Dawley rats (400-450g). At the beginning of each of the seven cycles, for 10 waveforms the mean and standard error was calculated for M-wave measurements from the 28 rats. Specifically, the M-wave amplitude, area, and duration were averaged. The nonparametric Friedman test was performed to determine any significant differences between the cycles for all the M-wave data. The Wilcoxin test was performed to identify which point(s) showed the greatest changes.

The rats were divided into their respective work/rest groups (table 4-1) and Wilcoxin tests were performed to determine the cycles that differed for each group. A comparison between each group using the Mann-Whitney Test was then performed. The groups were separated into duty cycle (DC) and cycle time (CT) groups (table 4-3) and the Wilcoxin and Mann-Whitney tests were repeated on these groups.

Table 4-3: Work/rest groups making up duty cycle and cycle time groups

25% DC	75% DC	20s CT	180s CT
25% DC, 20s CT	75% DC, 20s CT	25% DC, 20s CT	25% DC, 180s CT
25% DC, 180s CT	75% DC, 180s CT	75% DC, 20s CT	75% DC, 180s CT

The above analyses evaluated fatigue over the duration of the experiment. An assessment of the M-wave data from the beginning to the end of a cycle is another method of evaluating fatigue. Therefore, in addition to the averaged data obtained at the beginning of each of the 7 cycles, 10 waveforms were averaged at the end of those same cycles. Wilcoxin tests were performed comparing the

beginning and end of each cycle to determine if there was a significant change in the M-wave signal within each cycle. Also, Mann-Whitney tests were performed on the differences between the beginning and end of the cycle comparing each group, as well as the duty cycles and cycle time groups.

An alpha level of $p<0.05$ was considered significant for these analyses. All statistical procedures were performed in PASW Statistics v18.1 (SPSS, Inc Chicago, IL). The nonparametric tests were used due to non-normal distribution.

4.4.3 Results

Figure 4-6 shows the changes in M-wave amplitude, area, and duration for the pooled data (n=28) over the 6-hour stimulation period. In each variable significant change occurred as a result of the stimulation. Specifically, amplitude and area decreased and duration increased over the 6 hours.

The change in amplitude was immediate as the 0.05-hour cycle was significantly less than the initial cycle. This decrease in amplitude was maintained throughout the 6 hours as each of the remaining cycles were significantly less than the initial cycle. Also, the 2-hour cycle was significantly different from the 0.05-hour cycle. All other comparisons did not result in a difference.

The significant decrease in area occurred at 0.5 hours and continued to be significant throughout the 6 hours as each of the remaining cycles were significantly different from the initial cycle. Since the area at 0.05 hours was similar to the initial cycle, beginning at 1 hour, each of the cycles was significantly different from 0.05 hours. Also, the largest decrease in area occurred at 6 hours, resulting in this cycle being significantly different from 0.5 hours.

Duration increased significantly at 0.05 hours and remained elevated throughout the experiment. There was additional increase in duration at 2 hours as this cycle and the 4-hour cycle were significantly different from 0.5 hours. Also, a difference was observed between 4 hours and 0.05 hours.

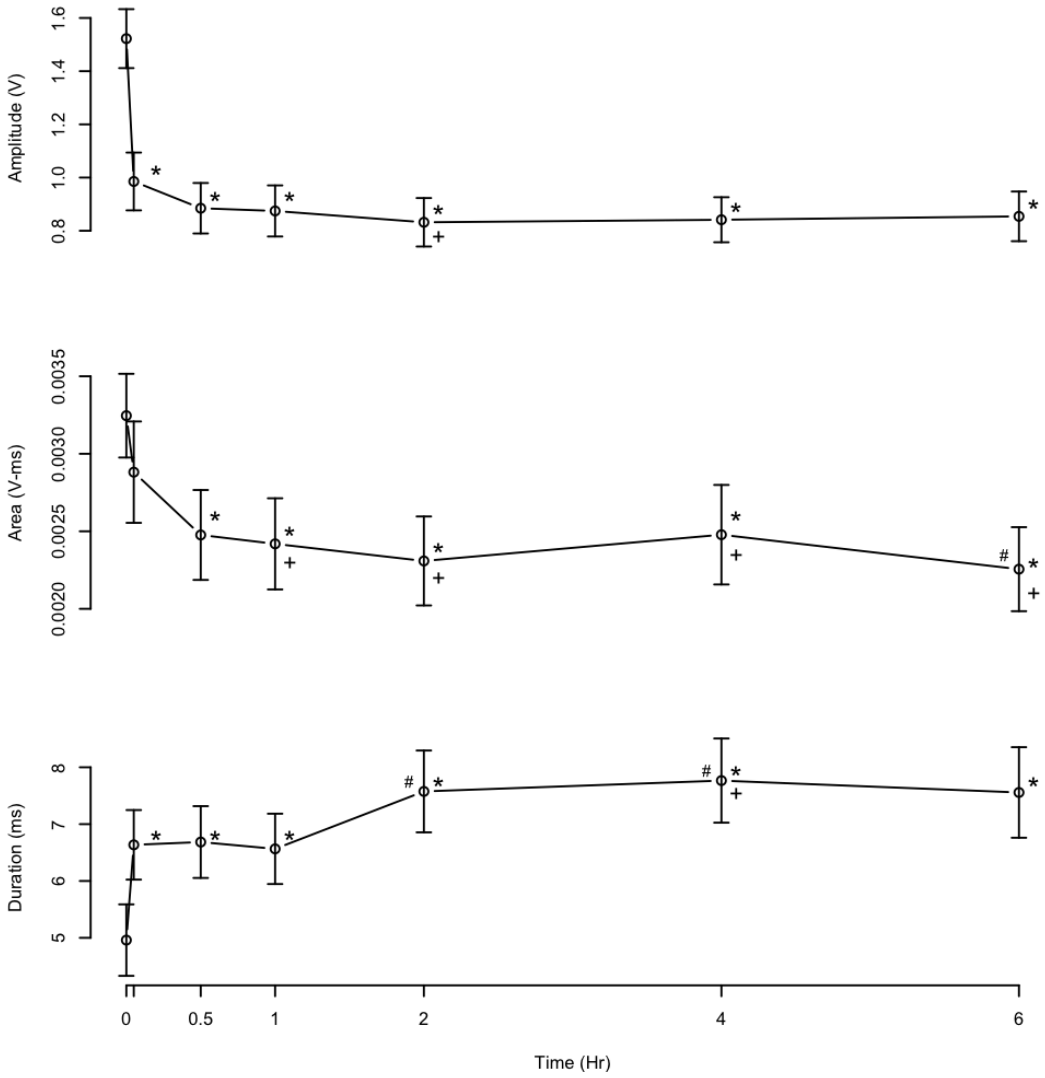


Figure 4-6: Overall average values of M-wave amplitude, area, and duration comparing each of the specified cycles throughout the 1-day experiment. Significant differences ($p<0.05$) are indicated by the following symbols: * different from the initial value, + different from 0.05hr, # different from 0.5hr.

4.4.3.1 Work/Rest Group Comparison Between Cycles

When separated into each work/rest group, the majority of the significant differences for amplitude occurred in comparison to the initial cycle in all groups except group D (DC 75% CT 180s). Figure 4-7 displays the trends in the changes in amplitude for each of the groups. Following the initial cycle, each of the group M-wave peaks decreased and remained below the initial cycle for the six-hour duration (figure 4-7). Group C (DC 75% CT 20s) had the largest amplitude decrease and it remained the lowest throughout the experiment compared to the other 3 groups. At 0.05 hrs the average amplitude in group C decreased to approximately 27% of the initial cycle amplitude. At 0.5 hr it rose to approximately 40% of the initial cycle amplitude and remained between 30% and 40% for the remainder of experiment.

Group B (DC 25% CT 180s) had the least amount of M-wave amplitude decrement at the initial time point. At 0.05 hrs, the amplitude decreased to approximately 90% of the initial cycle before decreasing slightly at 0.5 hrs to approximately 87% of the initial cycle and maintaining this amplitude until 1 hr. Another decrease was observed at 2 hrs to approximately 67% of the initial value, where it remained until the end of the experiment.

Groups A (DC 25% CT 20s) and D were the most similar in terms of initial amplitude decrement. By 1 hr the percentage amplitude was approximately 54% of the initial value. However, at 2 hours of stimulation the amplitudes began to diverge. While group A amplitude rose slightly to 56% of baseline, group D amplitude increased to 65% of baseline. The amplitude for group D continued to rise to 74% of baseline at 4 hrs and 80% of baseline at 6 hours. The amplitude

of group A fluctuated between 47% and 52% of baseline at 4 and 6 hours, respectively.

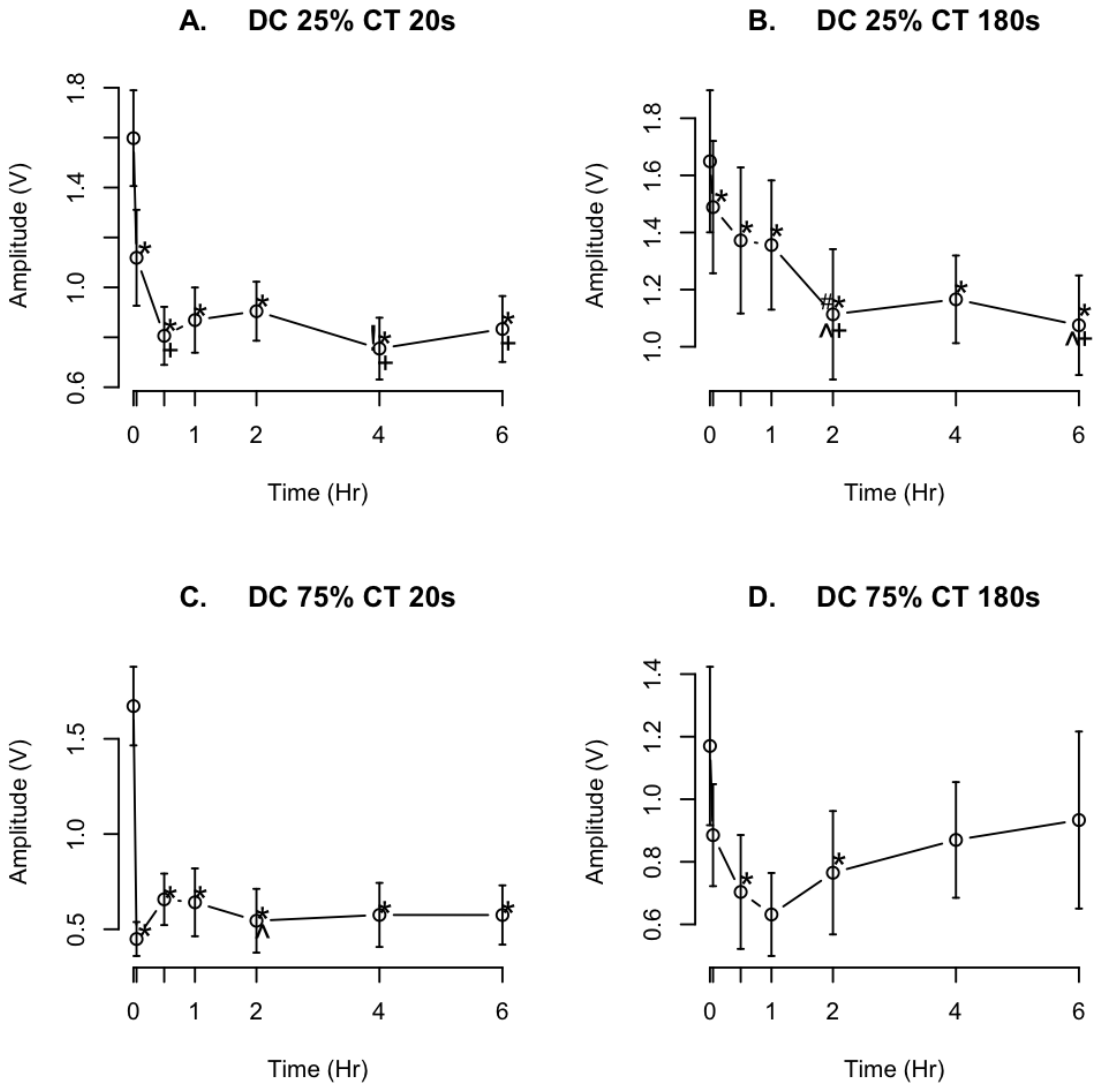


Figure 4-7: Average M-wave amplitude values comparison of the specified cycles throughout the 1-day experiment for each work/rest group. Significant differences ($p<0.05$) are indicated by the following symbols: * different from the initial value, + different from 0.05hr, # different from 0.5hr, ^ different from 1.0hr, ! different from 2.0hr.

Figure 4-8 displays the M-wave area results for each work/rest group. Group C resulted in the largest decrease in M-wave area from the initial to the 0.05-hour cycle. For each of the cycles M-wave area was significantly different

from the initial cycle for group C except the 1-hour cycle. The 3 other groups did not register a decrease at 0.05 hours. In fact, these 3 groups registered an increase in area at 0.05 hours before decreasing and remaining below the initial area for the remainder of the experiment.

Since an increase occurred at 0.05 hours, groups B and D had significant differences between later cycles and the 0.05-hour cycle. Group A only registered a significant difference between 6 hours and the initial cycle, while the area at 4 and 6 hours for group D began to increase. Group B had significant differences in M-wave area between the 0.05-hour and 2, 4, and 6-hour cycles.

The duration of the M-wave for each work/rest group increased at 0.05 hours and remained elevated throughout the 6-hour experiment (figure 4-9). The largest increase occurred in group A and this resulted in each of the cycles being significantly different from the initial cycle. Group A had the shortest initial duration at approximately 2ms, while the other groups began with about 5-6ms duration. The duration following the initial cycle for group A remained between 4 and 6ms, while the other group durations generally exceeded 7ms.

Group C had an initial rapid rise in duration at 0.05 hours and an additional rise at 2 hours, resulting in significant differences at 0.5, 4, and 6 hours compared to the initial cycle. Group B duration also had a significant rise at the 2-hour cycle and this resulted in a significant difference compared to 0.05, 0.5, and 1 hour. Group D initially increased in duration at 0.05 hours, resulting in a significant difference, decreased slightly at 0.5 hours before gradually increasing

for the remaining cycles, with the 2-hour cycle being significantly different than 1 hour.

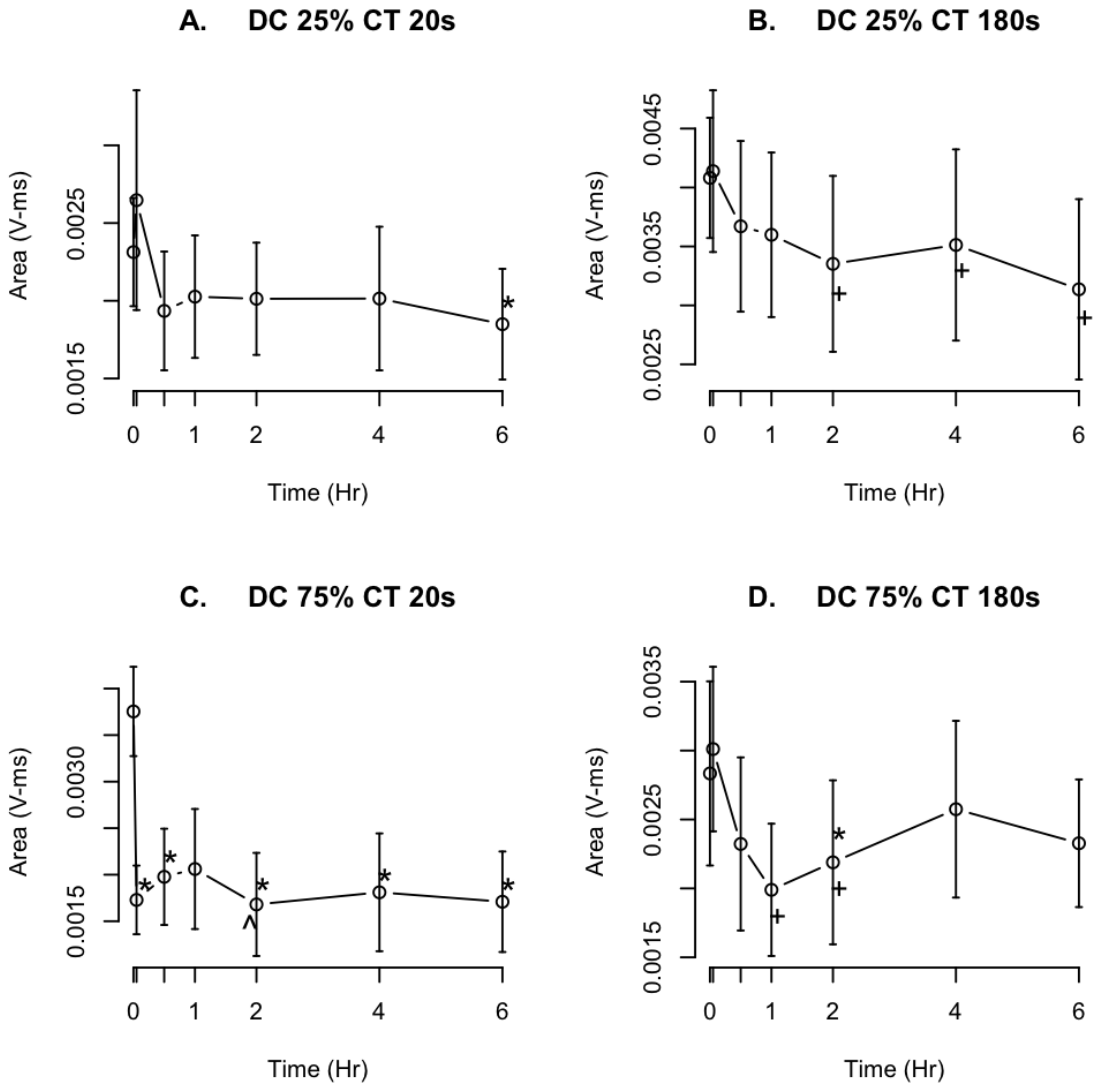


Figure 4-8: Average M-wave area values comparison of the specified cycles throughout the 1-day experiment for each work/rest group. Significant differences ($p<0.05$) are indicated by the following symbols: * different from the initial value, + different from 0.05hr, ^ different from 1.0hr.

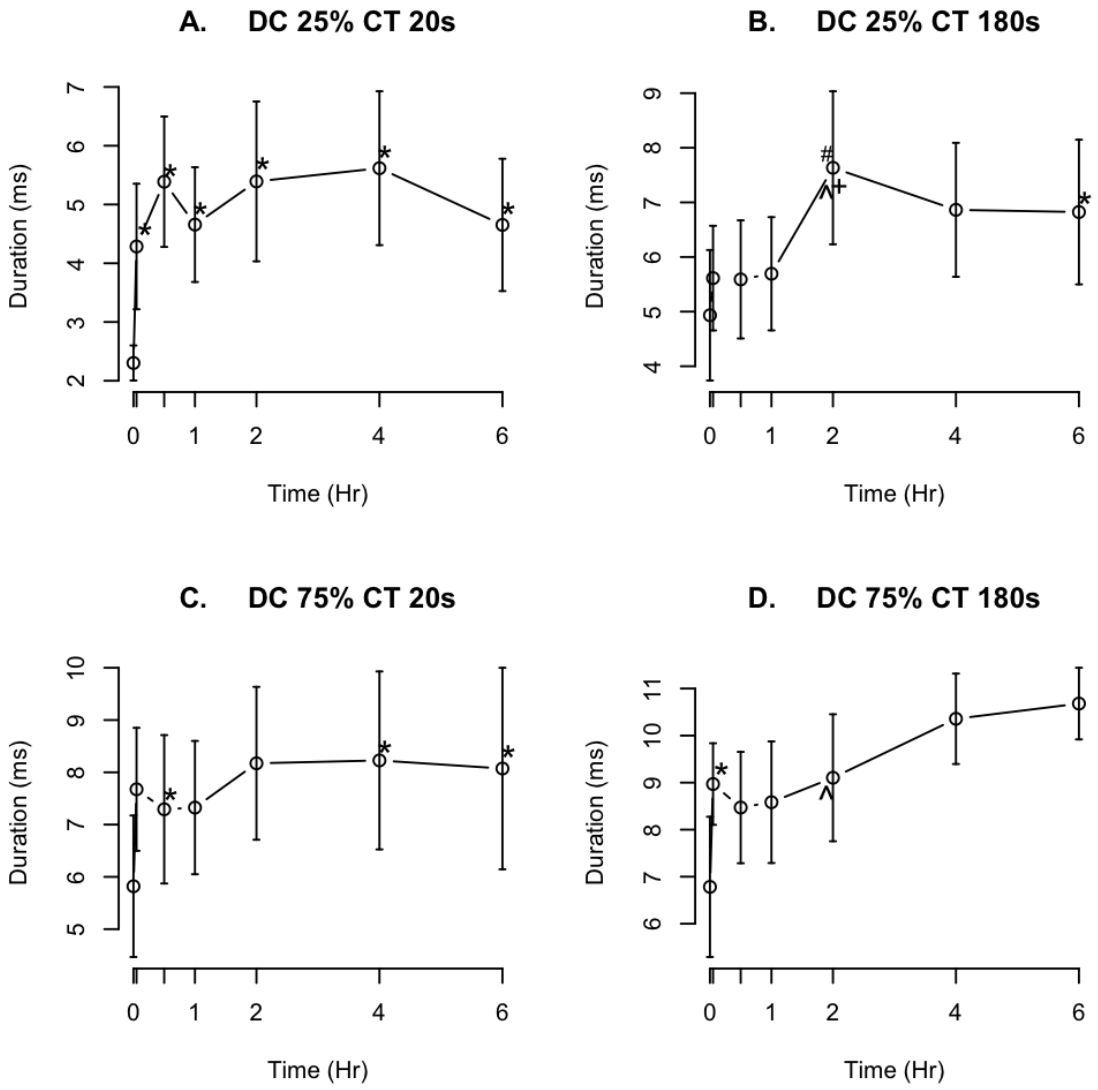


Figure 4-9: Average M-wave duration values comparison of the specified cycles throughout the 1-day experiment for each work/rest group. Significant differences ($p<0.05$) are indicated by the following symbols: * different from the initial value, + different from 0.05hr, # different from 0.5, ^ different from 1.0hr.

Mann-Whitney Tests, in which each work/rest group was compared to each other individually, was conducted on the amplitude, area, and duration data. Figure 4-10 displays the results in terms of group comparisons resulting in significant interactions. For the amplitude data, other than the comparison between groups A and C at 0.05 hr, groups B and C provide all the significant

differences. There was a significant difference at each cycle between groups B and C, indicating that duty cycle and cycle time influence the amount of fatigue from stimulation, as these two groups differed in both duty cycle and cycle time. Figure 4-10 indicates that the combination of a high duty cycle and short cycle time (DC 75% CT 20s) results in more fatigue than a low duty cycle and long cycle time (DC 25% CT 180s). In other words, the group with the shortest rest allowance fatigued much more than the group with the longest rest allowance.

In a comparison between the different groups for area, the M-wave area at 0.05 hours between groups B and C was significantly different. Another significant difference was found between groups A and B at 0.5 hours. M-wave area of groups A and B and A and C were also different at the initial cycle. The remaining comparisons between groups at each cycle provided no significant results. Figure 4-10 indicates that for area, the low duty cycle and long cycle time resulted in the least amount of fatigue.

The M-wave duration recorded for the rats in group A at the initial cycle was shorter than the other groups. This resulted in significant differences between group A and groups C and D. This trend continued at 0.05 hours, where group D was significantly different from group A. Also, group D was significantly different from group B. Group D maintained the longest duration at each cycle and was significantly different from group A at 1 hour and 6 hours, as well as being significantly different from group B at D and 6 hours. Duration of the M-wave was increased when muscles were stimulated at a high duty cycle and long cycle time (figure 4-10).

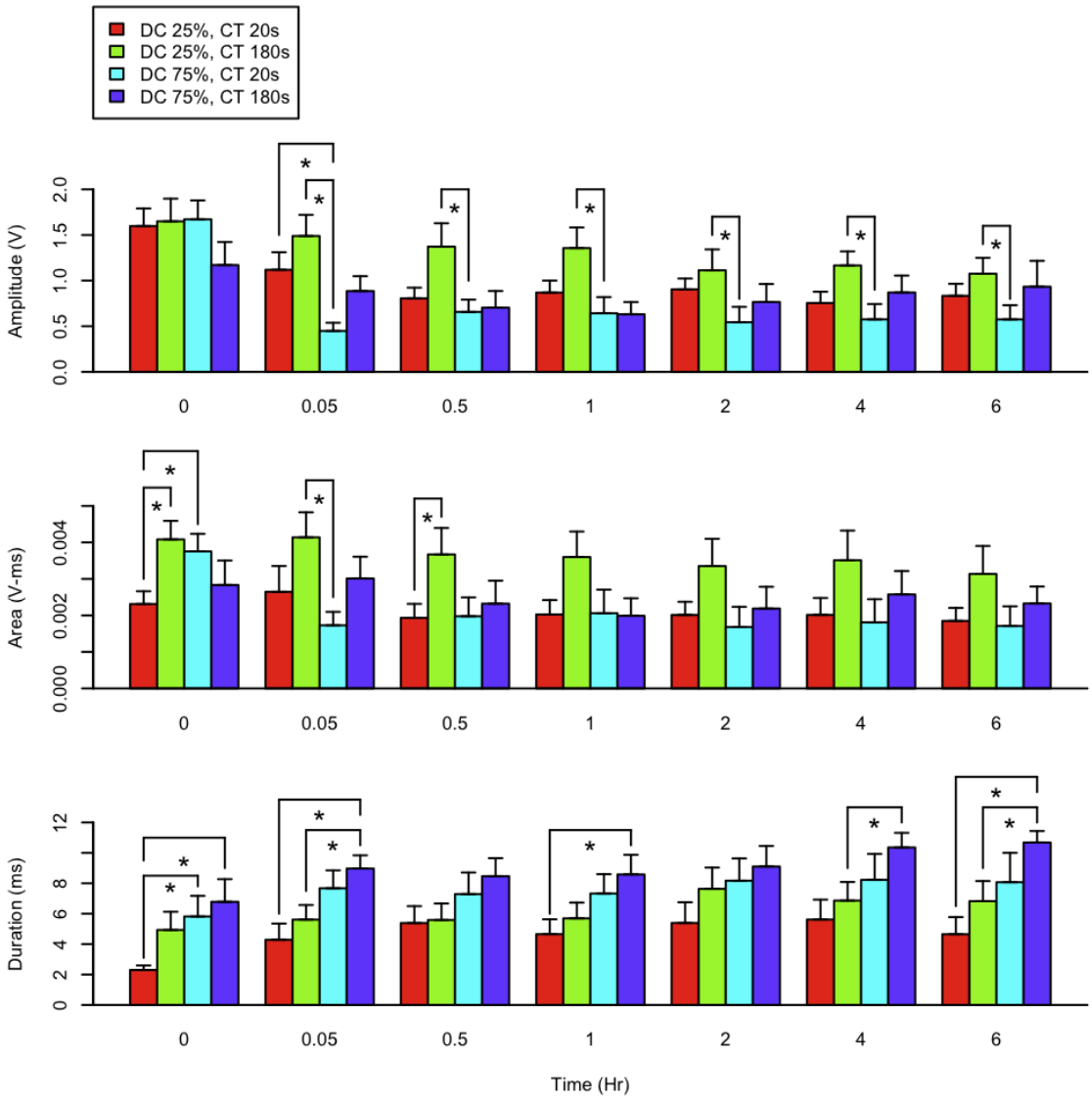


Figure 4-10: Average M-wave amplitude, area, and duration values comparison of each of the work/rest groups at the specified cycles throughout the 1-day experiment. Significant differences ($p<0.05$) are indicated by brackets and the * symbol.

4.4.3.2 Duty Cycle and Cycle Time Comparison Between Cycles

Table 4-3 provides the group combinations for each duty cycle and cycle time categories. The amplitude for both duty cycle groups and both cycle time groups decreased significantly between the initial and 0.05-hour cycle (figure 4-11). The amplitude was decreased for the remaining cycles and these were all significantly different from the initial cycles. The 75% duty cycle group and 20s

cycle time group had the steepest decrease in amplitude. The other groups, duty cycle 25% and cycle time 180s, had more gradual amplitude decline and similar amplitudes.

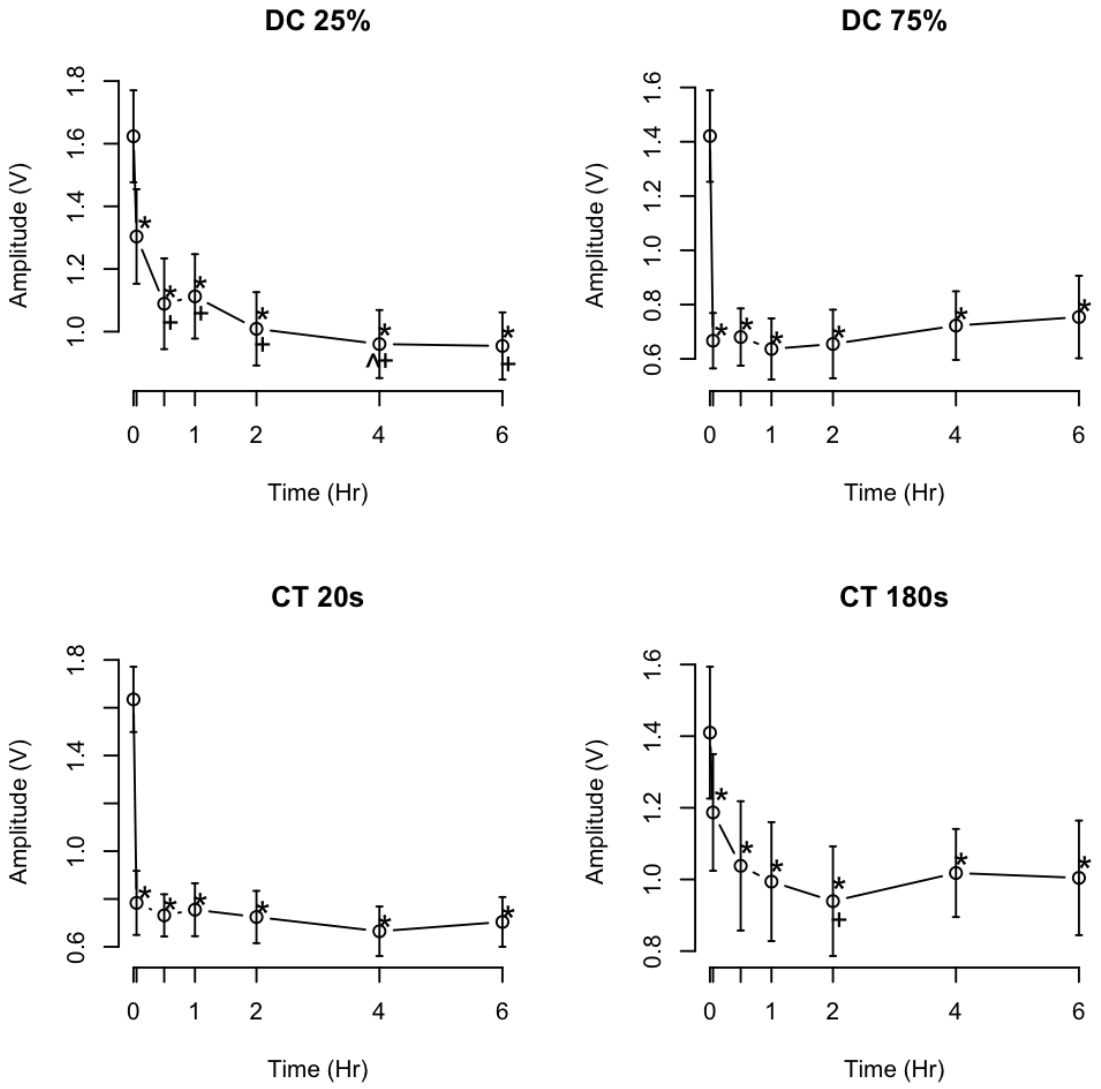


Figure 4-11: Average M-wave amplitude values comparison of the specified cycles throughout the 1-day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) are indicated by the following symbols: * different from the initial value, + different from 0.05hr, ^ different from 1.0hr.

The area decreases for the duty cycle and cycle time groups had similar trends to the amplitude decreases. The 75% duty cycle and 20s cycle time

groups had a larger decrease in area compared to the more gradual decrease of the 25% duty cycle and 180s cycle time groups (figure 4-12). Both of the latter groups had the area rise at 0.05 hours before decreasing. This resulted in all the cycles being significantly different from 0.05 hours except the 2-hour cycle for the 25% duty cycle group. Also, cycles 0.5, 1, 2, and 6 hours were significantly different from the initial cycle for these two groups. In the 75% duty cycle and 20s cycle time groups the M-wave area in all the cycles from 0.5 hours to the end of the experiment was significantly different from the initial cycle.

Duration for each of the duty cycle and cycle time groups increased at the 0.05 hour cycle and continued to be elevated for the remainder of the experiment (figure 4-13). This resulted in significant differences between all of the cycles compared to the initial cycle except for the 1 hour and 2 hour cycles for the 75% duty cycle group and 1 hour for the 180s cycle time group. A trend that occurred for all the duty cycle and cycle time groups was the additional increases in duration at the 2-hour cycle. Following the increase at 0.05 hours, the duration remained relatively constant at 0.5 hours and 1 hour before increasing again at 2 hours.

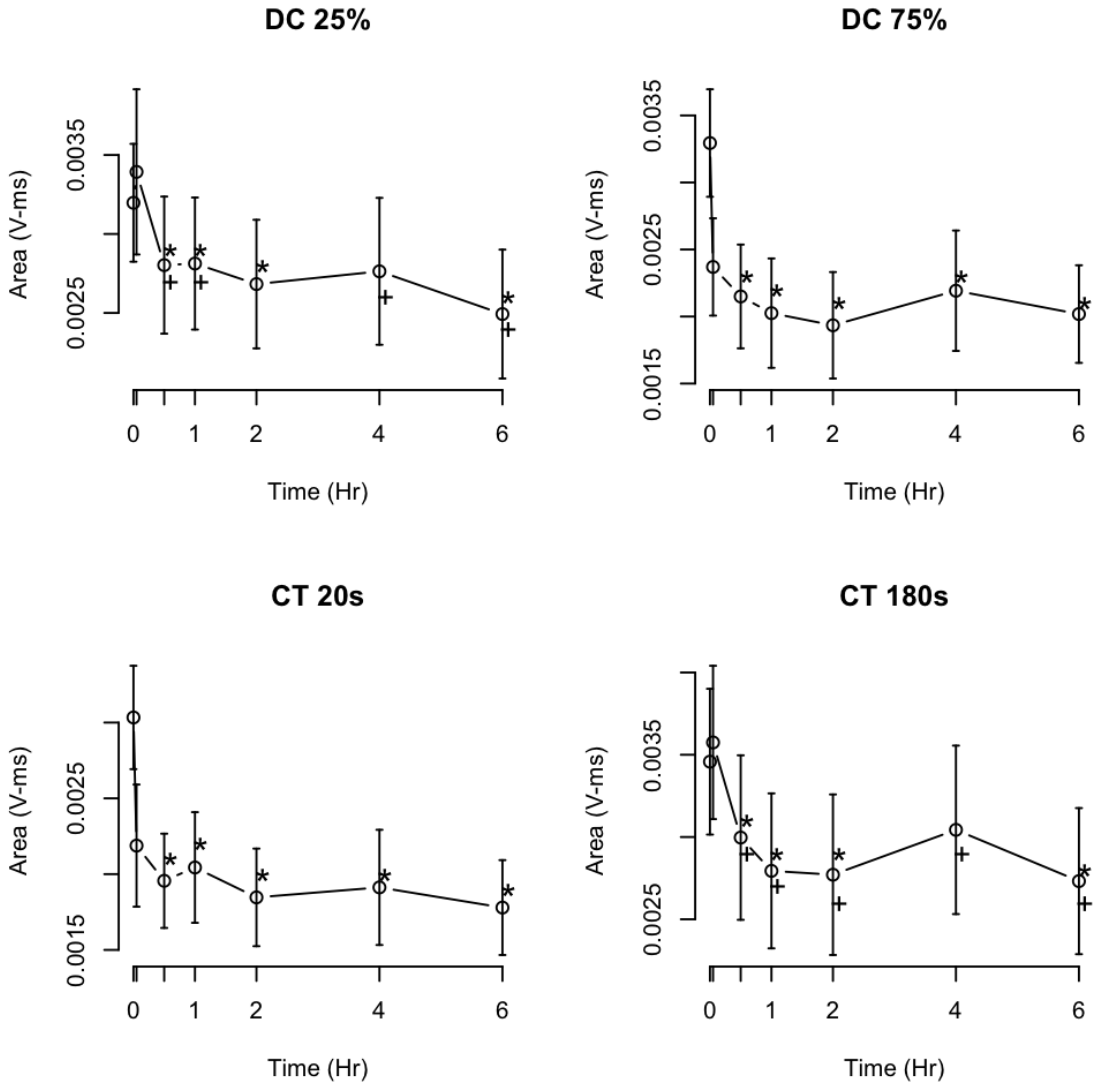


Figure 4-12: Average M-wave area values comparison of the specified cycles throughout the 1-day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) are indicated by the following symbols: * different from the initial value, + different from 0.05hr.

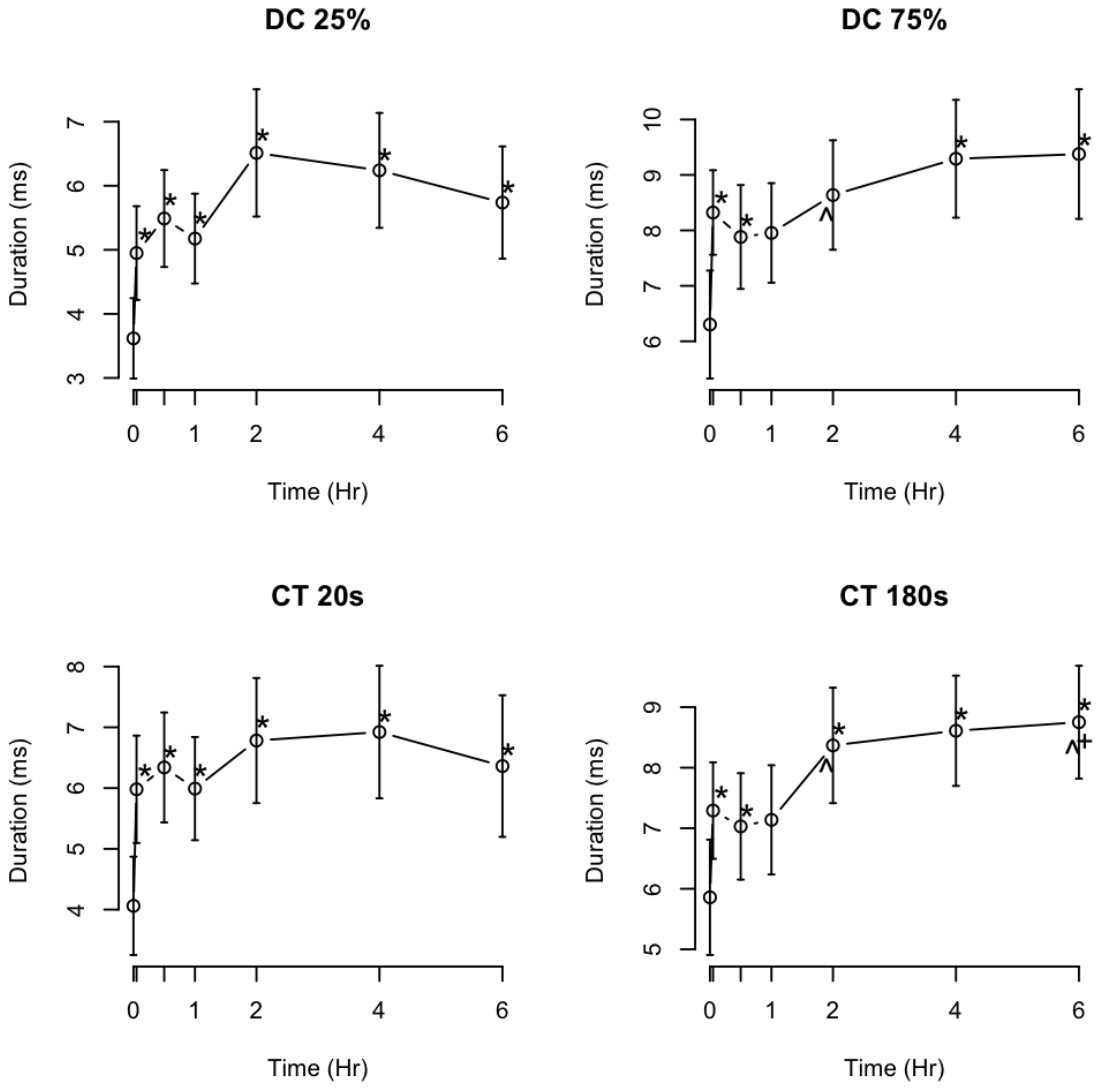


Figure 4-13: Average M-wave duration values comparison of the specified cycles throughout the 1-day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) are indicated by the following symbols: * different from the initial value, + different from 0.05hr, ^ different from 1.0hr.

A direct comparison was performed between the 2 duty cycle groups and between the 2 cycle time groups (figure 4-14). Mann-Whitney tests indicated that there were significant differences between duty cycle groups for amplitude and duration measurements, but not area. Specifically, there was a significant decrease in amplitude for the 75% duty cycle group at 0.05, 0.5, 1, and 2 hours

and duration increased significantly at 0, 0.05, 1, 4, and 6 hours compared to the 25% duty cycle group. Cycle time had significant differences only at the 4-hour cycle for amplitude and at the 0.05 and 4 hour cycles for area. Generally, the 20s cycle time resulted in decreased amplitude, area, and duration compared to the 180s cycle time group.

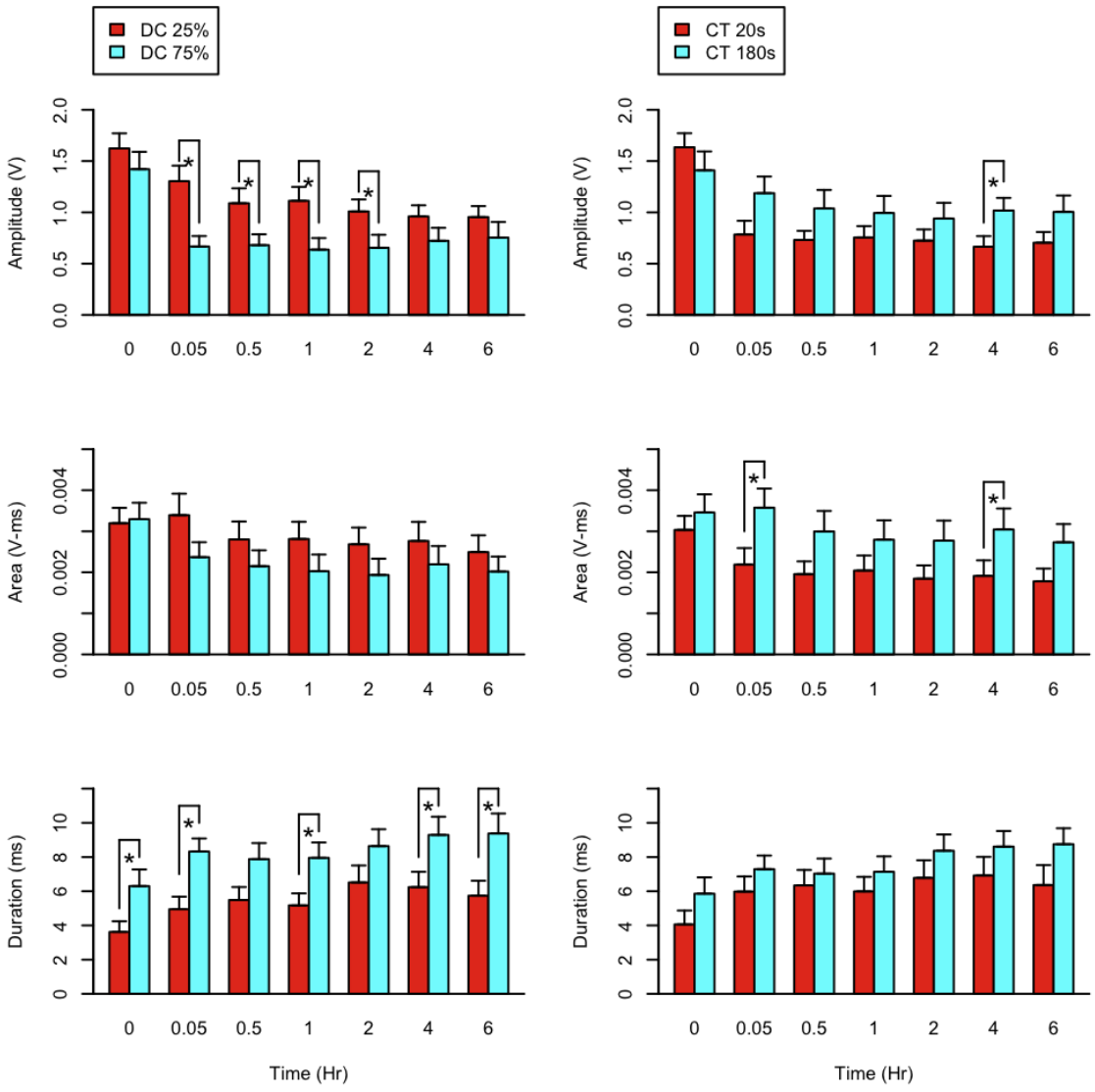


Figure 4-14: Average M-wave amplitude, area, and duration values comparison of each of the duty cycle and cycle time groups at the specified cycles throughout the 1-day experiment. Significant differences ($p<0.05$) are indicated by brackets and the * symbol.

4.4.3.3 Work/Rest Comparison From the Beginning to the End of Each Cycle

In addition to understanding the overall fatigue of each group of rats from the beginning to the end of the experiment, an analysis of fatigue was performed from the beginning to the end of each cycle. Figure 4-15 displays the pooled (n=28) results for the beginning and end of each cycle for M-wave amplitude, area, and duration. M-wave amplitude and area significantly decreased from the beginning to the end of each cycle. Duration of the M-wave increased from the beginning to the end of each cycle and was significant at the 0, 0.05, 0.5, and 1 hour cycles. The beginning of the amplitude and area cycles was always larger than the end of the previous cycle. The duration beginning at each cycle was always shorter than the end of the previous cycle.

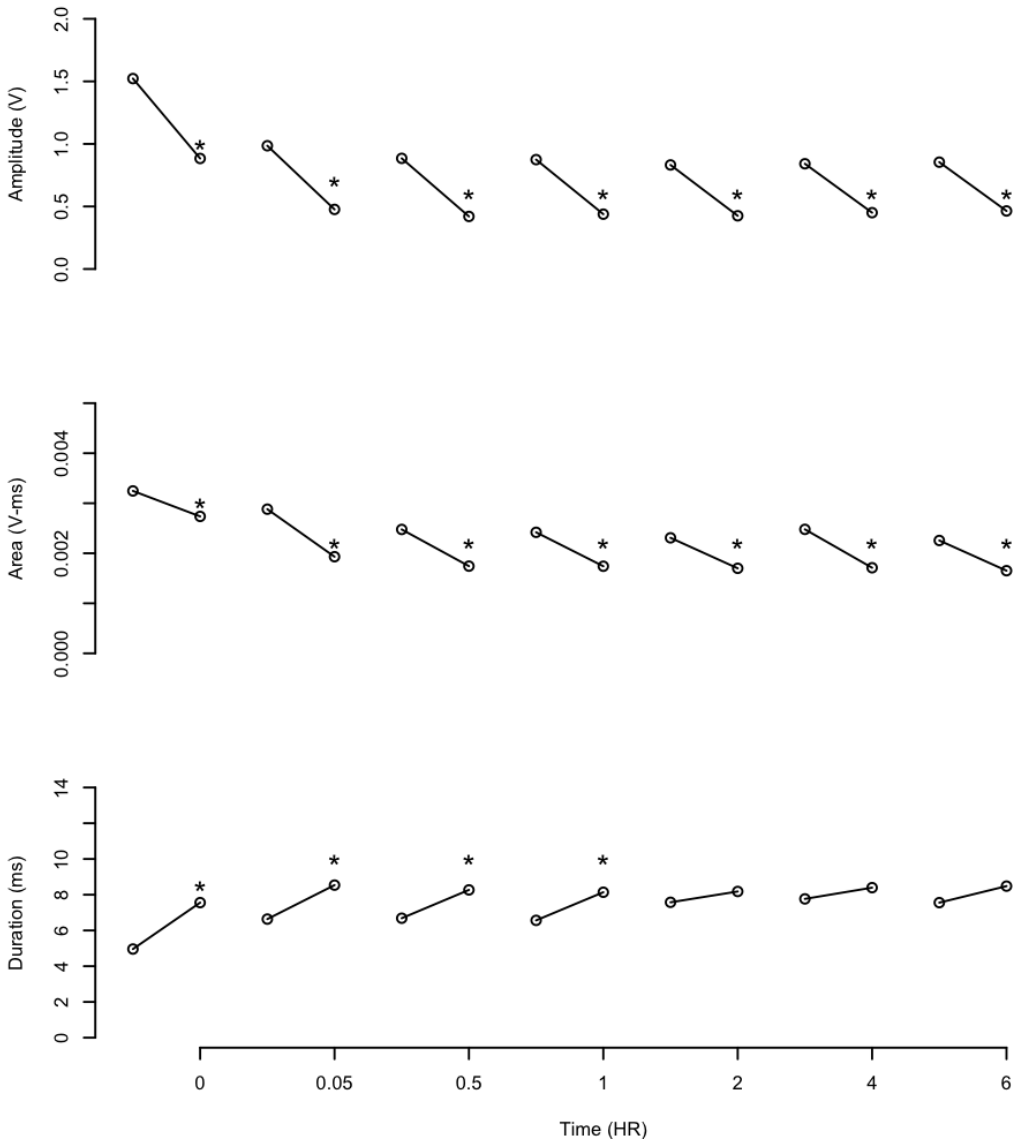


Figure 4-15: Overall average values of M-wave amplitude, area, and duration comparing the beginning to end of each of the specified cycles throughout the 1 day experiment. Significant differences ($p<0.05$) are indicated by the * symbol.

Figure 4-16 displays the comparisons between the beginning and end amplitude of each cycle for each work/rest group. Amplitude decreased from beginning to end of each cycle for each group. These decreases were significant for all of the cycles except the initial cycle for groups A and C. The M-wave amplitude at the beginning of each cycle was always greater than the end of the

previous cycle for all time points except 0.05 hours for groups A and C. Groups B and D had the largest decreases of M-wave amplitude and area between the beginning and end of each cycle. The beginning of the cycle had higher amplitude than the end of the previous cycle. Groups C and D had the lower end of cycle amplitudes than groups A and B.

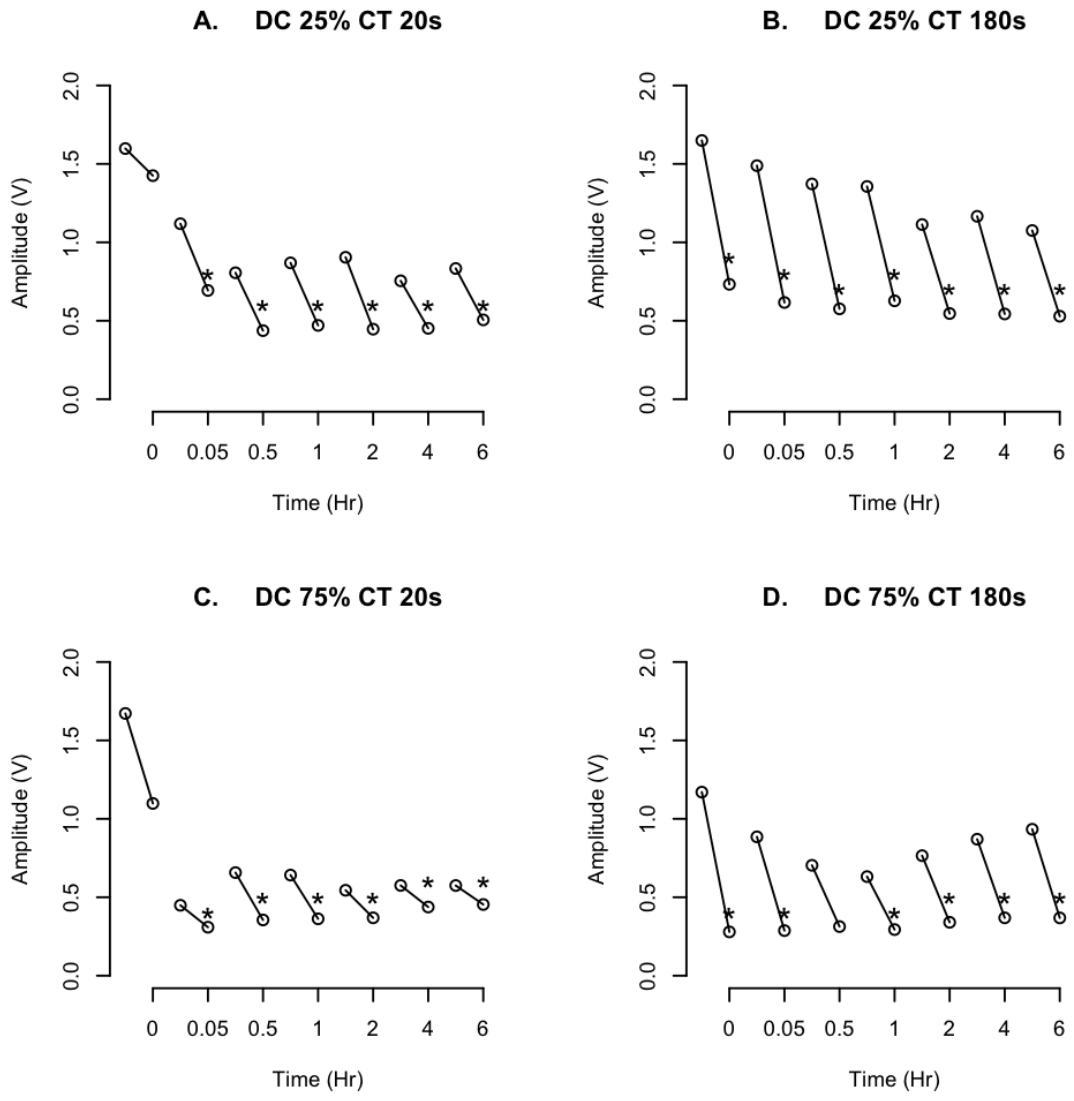


Figure 4-16: Average M-wave amplitude values comparison of the beginning to end of each of the specified cycles throughout the 1 day experiment for each work/rest group. Significant differences ($p<0.05$) are indicated by the * symbol.

The M-wave area in groups A and C initially increased from the beginning to the end of the initial cycle (figure 4-17). Group A's increase was statistically significant. Following the initial cycle, group A area decreased within the 0.05-hour cycle. At the beginning of the 0.5-hour cycle the area began at a lower level than the end of the previous cycle and continued to decrease until the end of the 0.5-hour cycle. M-wave area increased in the beginning of the 1-hour cycle and was followed by a pattern of decreasing area within the cycle and increasing area at the beginning of the following cycle. There were no significant differences from the beginning to the end of each cycle following the initial cycle.

After the initial cycle for group C, at 0.05 hours the M-wave area began the pattern of decreasing within a cycle and increasing at the beginning of the next cycle. The decrease from beginning to end of the cycle was significant for cycles 0.05, 0.5, 1, 2, and 4 hours.

Groups B and D followed a similar pattern, in which the area decreased from the beginning to the end of the cycle, followed by an increase at the beginning of the next cycle. This occurred at each cycle. For group B, this decrease in M-wave area was significant for every cycle, while it was significant only for the initial and 0.05-hour cycles in group D.

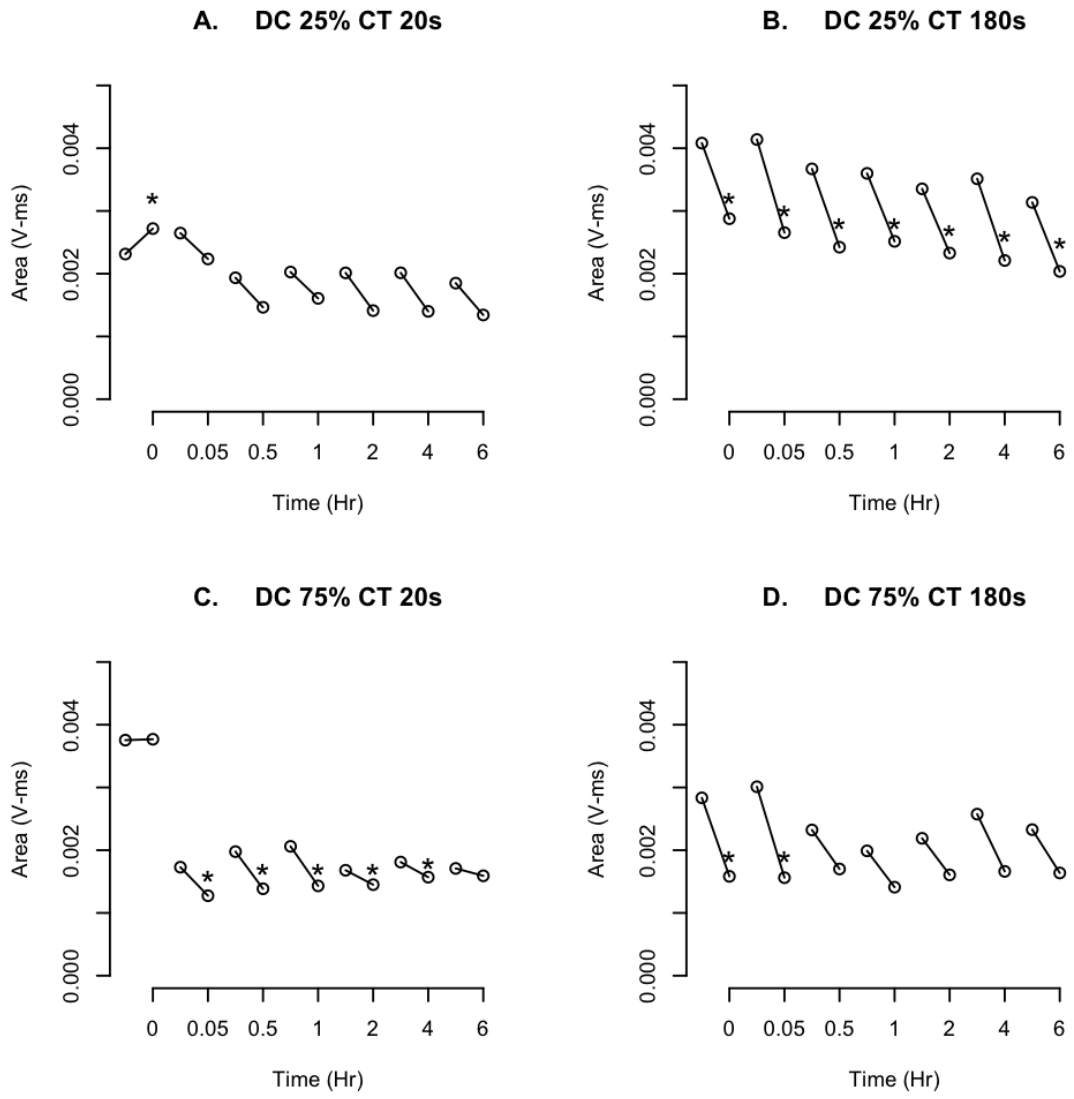


Figure 4-17: Average M-wave area values comparison of the beginning to end of each of the specified cycles throughout the 1 day experiment for each work/rest group. Significant differences ($p<0.05$) are indicated by the * symbol.

M-wave duration generally increased within each cycle for each group except group C (figure 4-18). There were no significant differences between the beginning and end of each cycle for groups C. M-wave duration in each cycle in group A increased from beginning to end of the cycles and the 0, 0.05, and 1-hour cycles were significant. M-wave durations in the beginning of each cycle

were shorter compared to the end of the previous cycles for each time point except at 0.05 hour. Group B had significant increases in duration within cycles for 0, 0.05, 0.5, and 1 hour. At 2 hours, duration decreased within the cycle. At 4 and 6 hours, duration again increased, but not significantly. Although M-wave durations for each of the cycles in group D increased from beginning to end of the cycle, only the initial cycle was significant.

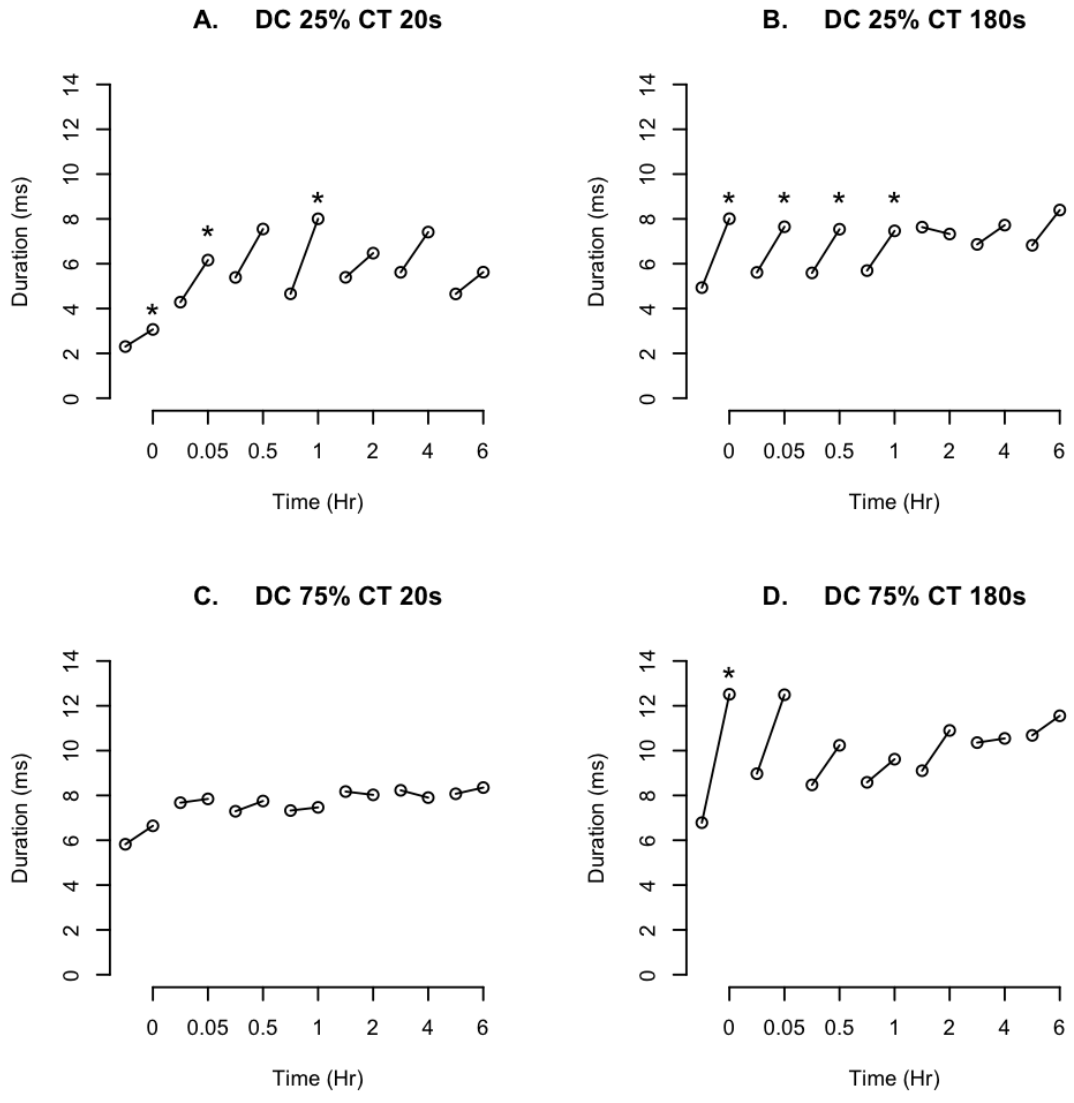


Figure 4-18: Average M-wave duration values comparison of the beginning to end of each of the specified cycles throughout the 1 day experiment for each work/rest group. Significant differences ($p<0.05$) are indicated by the * symbol.

The change in each M-wave measurement: amplitude, area, and duration were calculated to compare these changes between each group. This was done by subtracting the value at the end of the cycle from the beginning of the same cycle. A positive value for amplitude and area change was termed as a decrease, while a positive duration difference was an increase.

Group C resulted in the smallest decrease in amplitude and area and increase in duration from beginning to end of most cycles (figure 4-19). This decrease was significantly different than group B decrease for amplitude at 0.05, 1, 4, and 6 hours. At 0.05 hours, the amplitude decrease in group C was also significantly different than the decrease in groups A and D. Group A at 0 hours showed a significant difference in amplitude, area, and duration compared to groups B and D, and compared to group B for amplitude and area at 0.05 hours. Group A amplitude was also significantly different than group B at 6 hours.

The largest changes in M-wave area occurred within the initial and 0.05 hour cycles. Group C was significantly different from group B at 0.05 hours. Group A decreased significantly less in area compared to groups B and D at the initial cycle and group B at 0.05 hours.

There were significant differences in duration change in the initial and 0.05 hour cycle. At the initial cycle, groups B and D had a significantly greater increase in M-wave duration than group A. At 0.05 hours, groups B and C were significantly different. An additional difference was found between groups A and C at 4 hours.

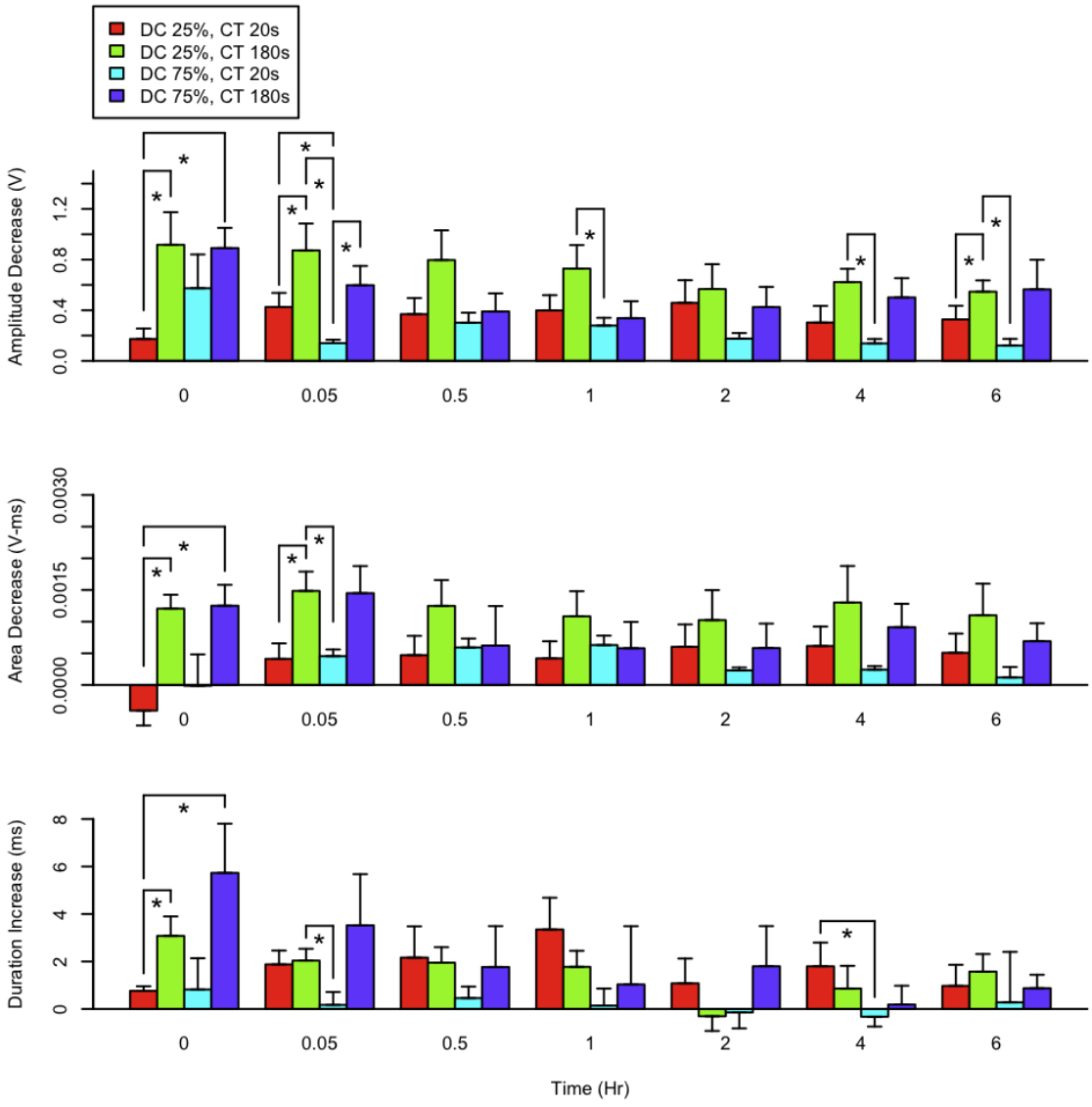


Figure 4-19: Comparison of each of the work/rest group's average M-wave amplitude, area, and duration difference from the beginning to the end of each of the specified cycles throughout the 1-day experiment. Significant differences ($p<0.05$) are indicated by brackets and the * symbol.

4.4.3.4 Duty Cycle and Cycle Time Comparison From the Beginning to the End of Each Cycle

The change in M-wave amplitude between the beginning and end of each cycle was significantly different for each duty cycle and cycle time groups at every cycle (figure 4-20). The 20s cycle time group at the initial cycle had a larger ending amplitude than the beginning amplitude of the 0.05 hour cycle. Following this cycle, each M-wave amplitude at the beginning of the cycle was higher than the M-wave amplitude at the end of the previous cycle. The 25% and 75% duty cycles had an ending amplitude at the initial cycle that was less than the beginning of the 0.05 hour cycle but M-wave amplitudes in these ending cycles were higher than in the remaining ending cycles. The 180s cycle time group had a change in amplitude that ended at a similar value throughout each cycle.

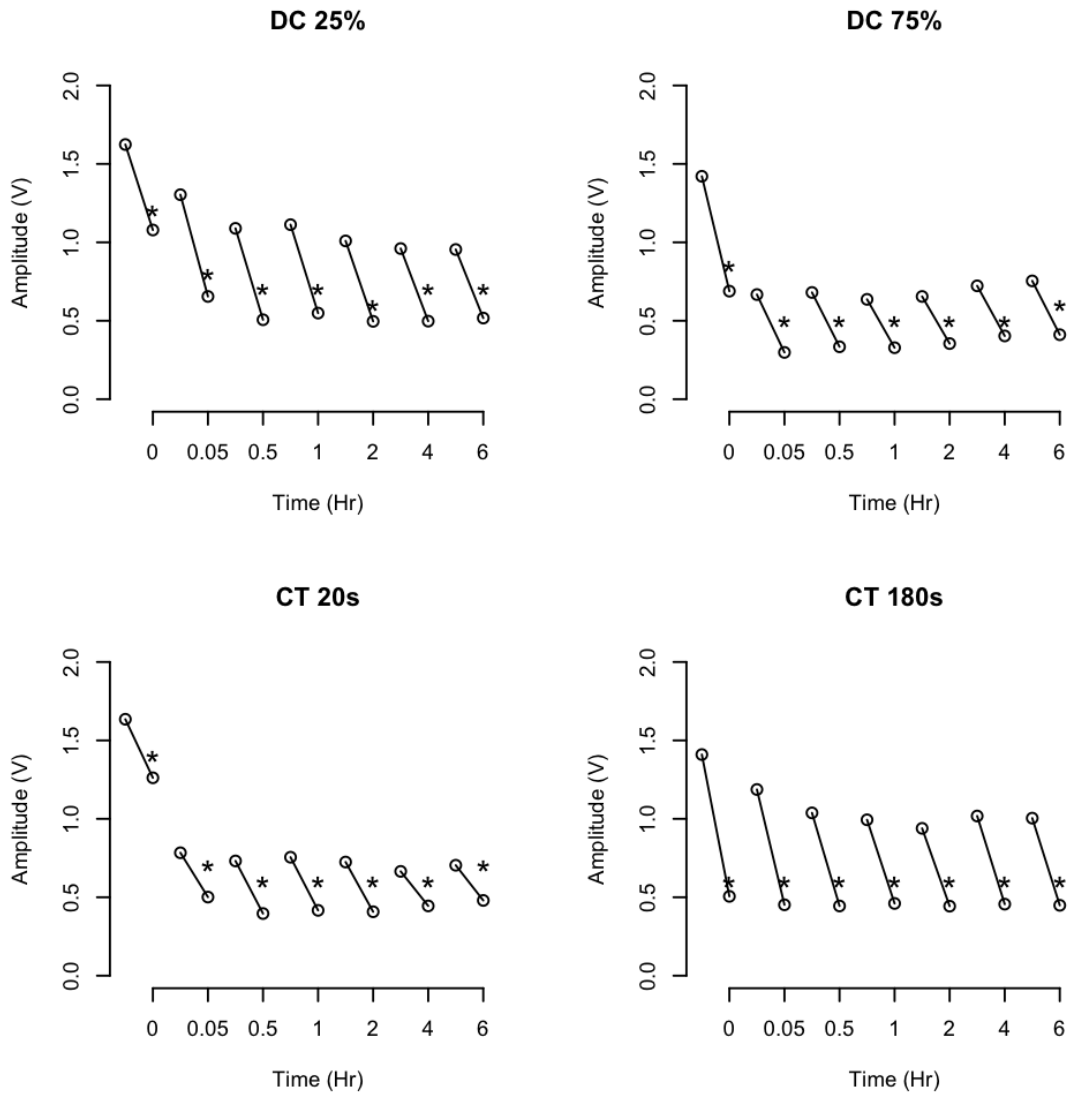


Figure 4-20: Average M-wave amplitude values comparison of the beginning to end of each of the specified cycles throughout the 1 day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) are indicated by the * symbol.

The change in M-wave area for each duty cycle and cycle time group were significant for each cycle except at the initial cycle for the 25% duty cycle and 20s cycle time groups (figure 4-21). At the initial cycle for the 20s cycle time group the area change from the beginning to the end of the cycle increased, where all other cycles for each group decreased. The area change at the end of the initial

cycle in the 75% duty cycle group was higher than at the beginning of the next cycle. The remaining areas at the end of each cycle were less than the beginning of the next cycle.

M-wave duration increased from the beginning to the end of each cycle for all duty cycle and cycle time groups at each time point (figure 4-22). These increases were significant for the 25% duty cycle group for each cycle except at 2 hours. Only the initial cycle resulted in a significant increase in duration from beginning to end of the cycle in the 75% duty cycle group. The 20s cycle time group resulted in significant increases at the initial, 0.05, and 1 hour cycles, while the 180s cycle time group had significant increases at the initial, 0.05, 0.5, and 6 hour cycles.

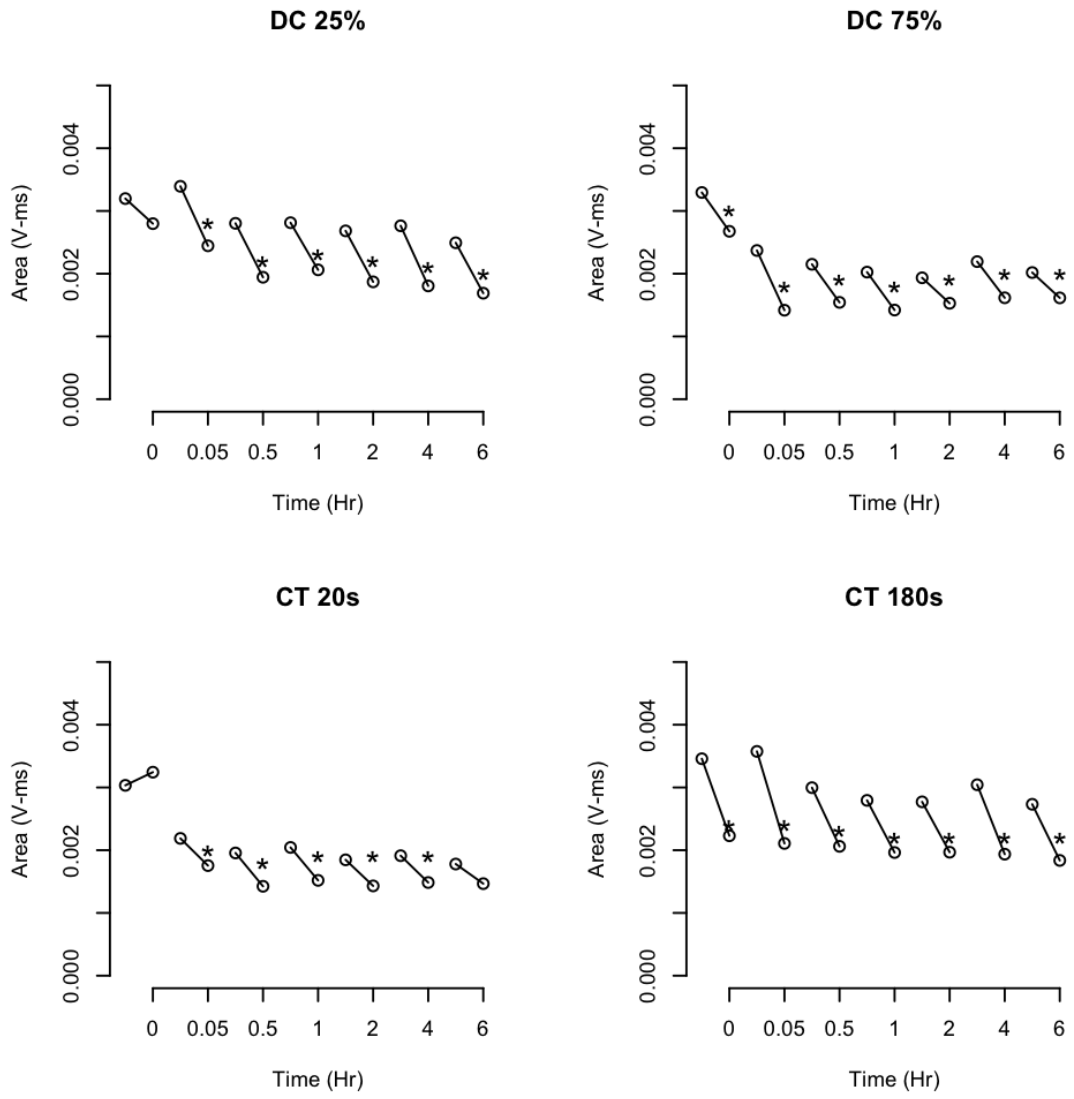


Figure 4-21: Average M-wave area values comparison of the beginning to end of each of the specified cycles throughout the 1 day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) are indicated by the * symbol.

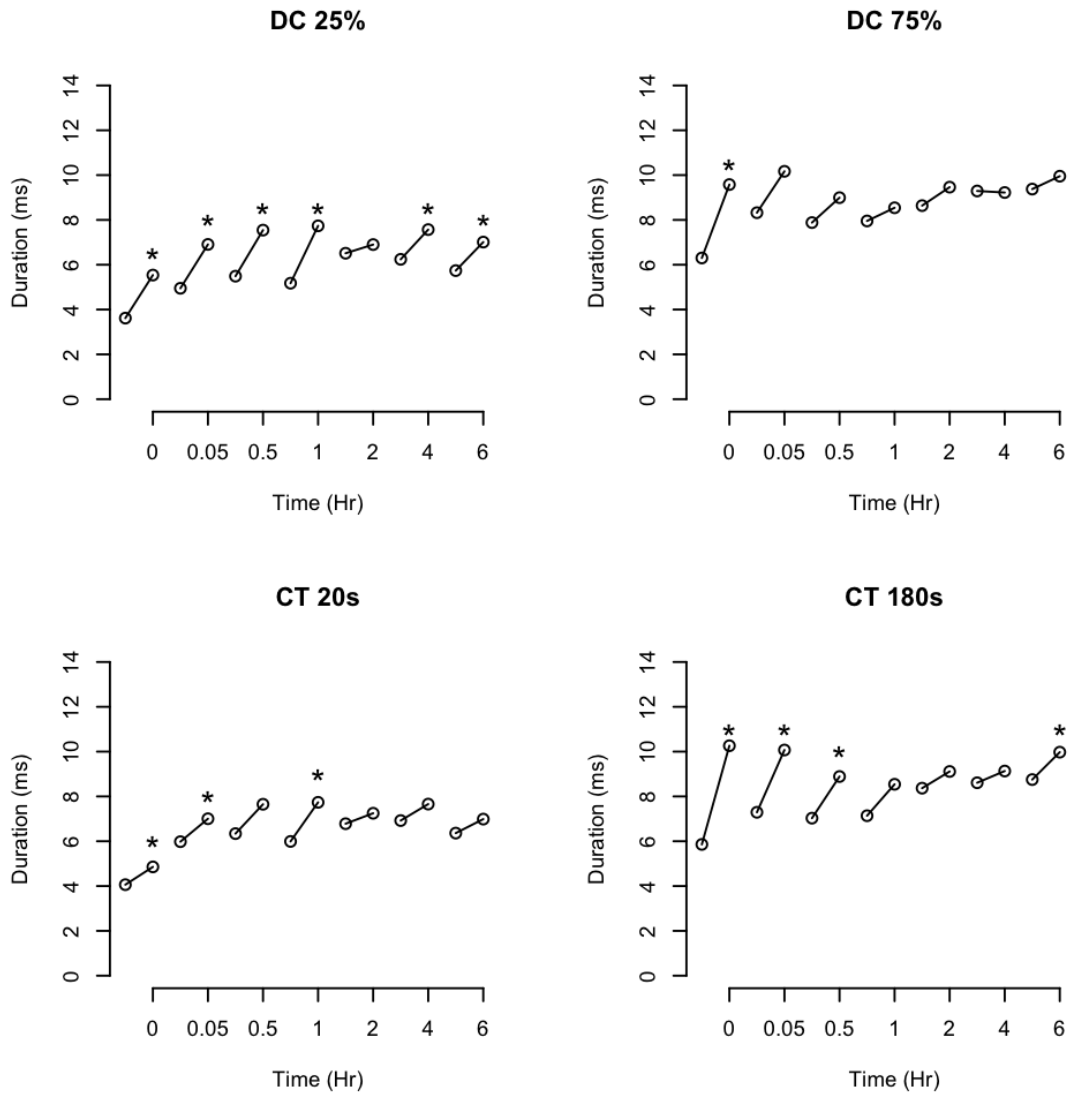


Figure 4-22: Average M-wave duration values comparison of the beginning to end of each of the specified cycles throughout the 1 day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) are indicated by the * symbol.

The initial cycle amplitude, area, and duration changes were greater for the 75% duty cycle compared to the 25% duty cycle (figure 4-23). The cycles at 0.05, 0.5, 1, 2, 4, and 6 hours had a larger change in M-wave amplitude, area, and duration for the 25% compared to the 75% duty cycle. Only the changes at

0.05 hours for amplitude and 1 hour for duration resulted in significant differences between the 25% and 75% duty cycle groups.

Cycle time had slightly more effect on the changes observed between the beginning and end of each cycle. The change in M-wave amplitude, area, and duration were greater in the 180s cycle time group compared to the 20s cycle time group in all cases except for the 1 hour and 4 hour duration cycles. The increased amplitude in the 180s cycle time group was significant at the initial 0.05, 4, and 6 hour cycles. Areas at the initial and 0.05 hour cycles also were significantly different, while duration had a significant difference at the initial cycle.

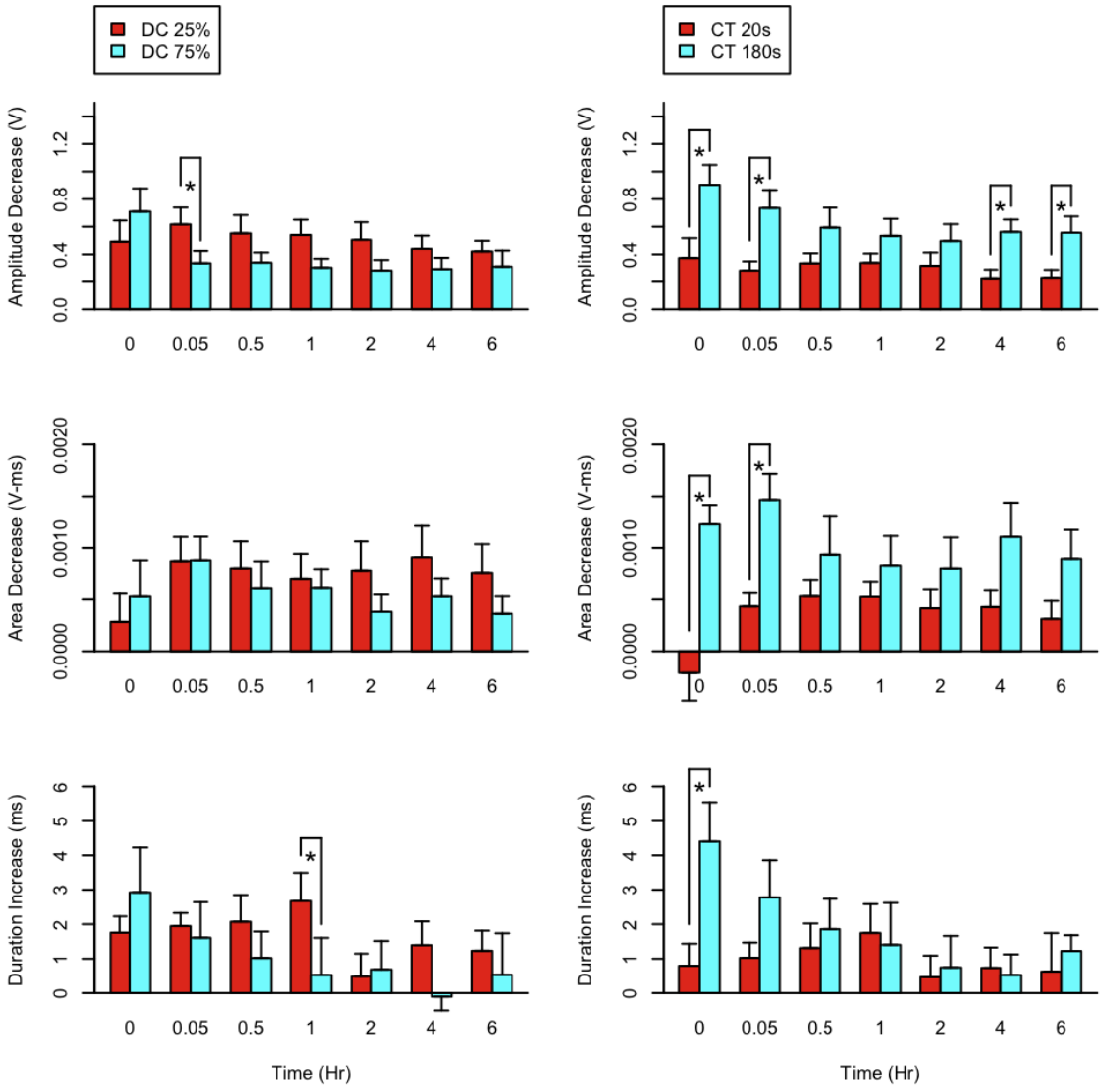


Figure 4-23: Comparison of each of the duty cycle and cycle time groups average M-wave amplitude, area, and duration difference from the beginning to the end of each of the specified cycles throughout the 1-day experiment. Significant differences ($p<0.05$) are indicated by brackets and the * symbol.

4.4.4 Discussion

Electrical stimulation had an immediate effect on muscle fatigue in these studies. In each of the 4 groups, longissimus muscle M-wave amplitude and area decreased and duration increased within the first cycles of stimulation and were measured at the 3-minute cycle. These M-waves never returned to their

original values throughout the experiment. They appeared to settle at a specific baseline value and maintained that level. However, the amount of change in the parameters and hence, the degree of fatigue differed between the groups.

The groups were separated into the following: group A (DC 25% CT 20s), group B (DC 25% CT 180s), group C (DC 75% CT 20s), and group D (DC 75% CT 180s). The combination of 75% duty cycle 20s cycle time had the most profound effect on the M-wave measurements. At the 3-minute cycle, the amplitude and area of group C had the greatest decrease and largest increase in duration compared to the other groups. In fact, area increased for the other 3 groups at 3 minutes before decreasing. This decrease in amplitude was significantly different from groups A and B but not group D. Since groups A and B have a 25% duty cycle and groups C and D have a 75% duty cycle, it seems that duty cycle and not cycle time had the greatest effect on M-wave amplitude. Thus, a higher percentage work/rest ratio increased the fatigue effects, regardless of the cycle time. This was confirmed in the analysis of duty cycles and cycle times, as there were significant differences at several time points between the 25% and 75% duty cycles in the amplitude and duration measures, while cycle time had only one significant difference in amplitude and 2 in the M-wave area measures.

The M-wave amplitude of the 75% duty cycle, 20s cycle time group remained lower than the other 3 groups for the entire duration of the experiment. This was supported by the 75% duty cycle group maintaining the lowest amplitude, followed closely by the 20s cycle time group. Stimulating at a 25%

duty cycle with a cycle time of 180s had the least effect on fatigue, based on the amplitude and area measurements. The decrease in amplitude and area occurred slowly, such that it did not reach a steady state baseline until the 2-hour cycle. This baseline level was higher than the other 3 groups.

Groups B and C M-wave amplitude measures were significantly different from each other at each cycle. These 2 groups differed in each duty cycle and cycle time categories. The protocol for group B represents short duty cycle with a long cycle time, while group C has a long duty cycle with a short cycle time. The primary difference between these 2 groups is rest allowance. Each cycle for group C allows for only 5 seconds of rest, while group B rats, even though the amount of stimulation time was longer, had 135 seconds of rest. The decreased rest allowance significantly reduced the M-wave amplitude and area compared to the smaller duty cycle and longer cycle time. It did not allow time for the M-wave to recover.

Duration of the M-wave did not follow the same trends as amplitude and area. It was found that group D, the protocol with the high duty cycle and long cycle time, had the largest duration measures. This duration did not reach a steady state baseline, but continued increasing throughout the 6-hour experiment. Duration is related to conduction velocity, while amplitude and area are determined by the size of the action potential (Bigland-Ritchie, 1981). This suggests that increasing duty cycle with a longer cycle time progressively decreases conduction velocity, regardless of the strength of the signal.

In addition to assessing the changes in M-wave parameters over the length of the experiment, an analysis was performed to determine the effects of stimulation on muscle fatigue within each cycle. Thus, the M-wave analyses were performed at the beginning and end of each specified cycle. Again, groups B and C resulted in differences in the M-wave amplitude and area measures. In group B, the amplitude and area at the end of each cycle was significantly different compared to the beginning. Because of the long cycle time, at 25% duty cycle, the amount of stimulation was 45s, resulting in the significant decrease in amplitude and area at the end of the cycle. With that long cycle time, the rest time allowance was 135s, resulting in an almost complete recovery of the M-wave at the beginning of the cycle.

While for the most part, the group C M-wave amplitude and area were significantly different at the end of each cycle compared to the beginning, the recovery of the amplitude and area did not approach the initial values. This was most likely due to the short rest allowance. Rather, the M-wave amplitude and area returned to the baseline established at the 3-minute cycle.

Comparing groups B and C amplitude decreases resulted in significant differences at most of the cycles. The differences between these 2 groups were most likely due to cycle time and not duty cycle. A comparison between the duty cycle and cycle time groups revealed significant differences in amplitude at 4 time points for the cycle time groups as opposed to 1 for the duty cycle groups. There appeared to be a greater effect on fatigue within a cycle due to cycle time, while over the course of the experiment, duty cycle played a larger role.

The changes in M-wave duration did not follow the same pattern as M-wave amplitude and area changes. Generally, duration increased from the beginning to the end of each cycle and these increases were significant in groups A, B, and D in the early cycles. The increases in M-wave duration were the smallest in group C and none were significant. The comparison between groups found group D at the initial cycle to have the largest increase in duration and this was significantly different from groups A and B. Again, a higher duty cycle with a longer cycle time slowed the conduction velocity, independent of the strength of the action potentials. An analysis of duty cycles and cycle times revealed cycle time was probably the influencing factor in the M-wave duration, as there was a significant difference in duration for cycle time at the first time point. The remainder of the experiment did not have an influence on duration changes from the beginning to the end of each cycle.

The fatigue of longissimus muscle that occurred in these *in vivo* rat experiments was most likely due to neuromuscular transmission failure, due to the changes in the M-wave. There was an immediate decrease in the amplitude and area, while duration increased from the beginning to the end of each cycle. There was recovery of this signal in the subsequent cycles, although not complete. This incomplete recovery may have injury implications, according to the proposed chronic injury model in figure 1-4 by Barr and Barbe (2004). The restoration of the ionic balance capacity of the tissue has been reduced and if the requirements are at the previous level, the muscle tissue may not be able to match those requirements, which can result in injury.

The results of this study were in accordance with Rhomert's (1973) work, which found that longer isometric contractions required longer rest allowances. This study also agreed with Iridiastadi and Nussbaum (2006) in that a high duty cycle produced greater fatigue in human deltoid muscles. The M-wave amplitude and area for the higher duty cycle groups in the current study decreased more than the lower duty cycle groups. The decreased M-wave amplitude and area suggests that there was a decrease in the number of action potentials (Bigland-Ritchie, 1981).

Iridiastadi and Nussbaum (2006) suggested a shorter cycle time would reduce the fatigue effect. Sundelin (1993) also found that frequent rest breaks with an increased work pace would be beneficial to the worker. In the current study, there were no significant differences in M-wave amplitude and area between the 2 groups with the high duty cycles. In other words the M-wave amplitude and area did not indicate that with a high duty cycle there was an advantage of a short or long cycle time. However, there was consistently a longer M-wave duration in the longer cycle time group. M-wave duration reflects the conduction velocities of the range of muscle action potentials (Bigland-Ritchie, 1981). The results suggest that a prolonged contraction slows the conduction velocity of the action potential, indicating increased fatigue in the longer cycle time group.

4.5 Evaluation of the stability of the EMG signal over several days

4.5.1 Specific Aim III

The previous 2 studies (sections 4.3 and 4.4) established the viability of electrically stimulating and recording from the medial longissimus and determined the occurrence of fatigue over a period of 6 continuous hours. Muscle injury due to repetitive motion most likely results from multiple days of exposure to a task. Thus, an evaluation of fatigue over several days may provide insight into this injury process.

During the entire 6-hour study the rats were anesthetized, reducing the chance of electrode movement within the muscle. Also, the electrode implantation method was selected to reduce movement artifact. In a multi day study, there will not be a luxury of continuous anesthesia, potentially affecting the electrode stability. Thus, the purpose of this study was to determine the stability of the electrodes by evaluating the M-wave signal at the beginning of each experimental day compared to the implantation day.

4.5.2 Statistical Analysis

On the day of surgery, after the electrodes were implanted, a brief stimulation was administered to a group of 24 rats and the EMG was recorded. The surgery to implant the electrodes was performed 5 days prior to the start of the first experimental day. The rats were then stimulated for 2 hours each day for 3 days (see table 4-4). The mean amplitude, area, and duration were calculated for 10 waveforms on the surgery day and on the beginning of the first cycle for each experimental day for all of the rats combined (n=24). A comparison between this surgery day recording and each of the experimental

day's initial recordings was performed using the Mann-Whitney test to determine if there was a consistent signal. All statistical procedures were performed in PASW Statistics v18.1 (SPSS, Inc Chicago, IL).

Table 4-4: Electrical muscle stimulation protocol work/rest ratios

		Cycle Time	
		20 seconds	**180 seconds**
Duty Cycle	**0.25 (25%)**	5 seconds stimulation 15 seconds rest n=6	45 seconds stimulation 135 seconds rest n=6
	0.75 (75%)	15 seconds stimulation 5 seconds rest n=6	135 seconds stimulation 45 seconds rest n=6

4.5.3 Results

The M-wave amplitude and area on the day of surgery were greater than the amplitudes and area at the initial cycle for each of the 3 experiment days (figure 4-24). Also, M-wave duration on the day of surgery was shorter than the duration at the initial cycle for each of the 3 experiment days. The M-wave amplitude, area, and duration for the initial cycle on each day were significantly different compared to their values on the day of surgery. There were no significant differences between each of the experiment days.

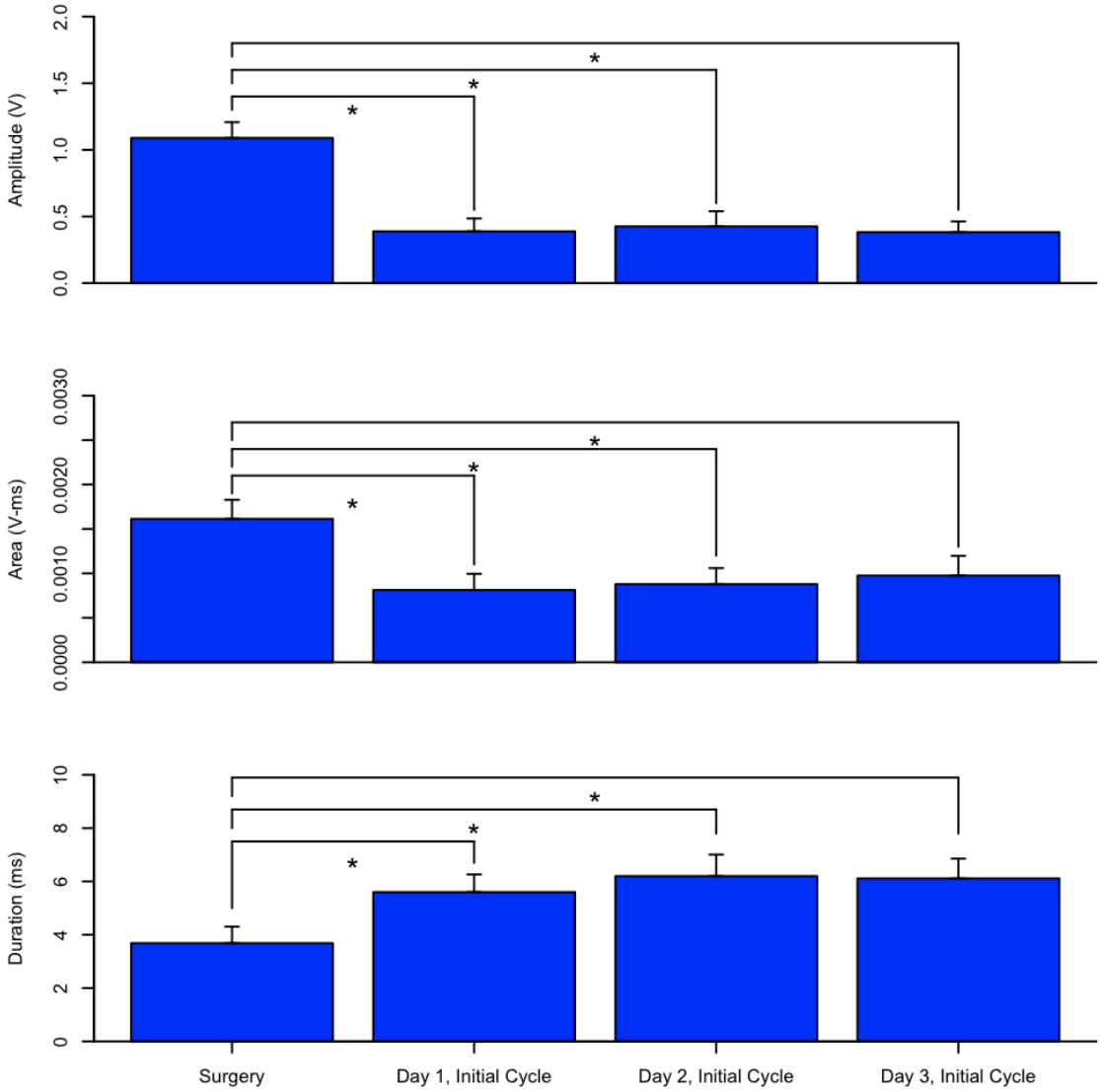


Figure 4-24: Average values of M-wave amplitude, area, and duration comparing the first cycle for each of the 3 experimental days and the day of the surgery. Significant differences ($p<0.05$) are indicated by brackets and the * symbol.

4.5.4 Discussion

Several studies have been performed in which stimulating electrodes have been implanted over several days (Egginton and Hudlicka, 2000; Eken and Gundersen, 1988; Hudlicka et al., 1994; Takahashi and Hood, 1993). The primary method of assessing changes in the physiological effects due to

stimulation was through histological analyses. Properties of the stimulus pulse were not measured over time in these studies.

Electromyography electrodes have also been implanted over periods of days or longer (Whelan, 2003). Generally the researchers have focused on the voluntary muscle contraction and not on stimulated muscle response. Voluntary contractions result in a random looking EMG signal due to the unsynchronized muscle fiber activation, while electrical stimulation produces a synchronized response, resulting in an M-wave. In long-term EMG studies, investigation into the stability of the EMG signal has not been performed.

In previous electrical stimulation studies carried out over several days, the experiment is begun days after the surgery was performed to implant the electrode (Eken and Gundersen, 1988). This is done to allow the animal to recover from the surgical procedure. Thus, this study allowed for a 5-day recovery between the surgical procedure to implant the electrode and the beginning of the experimental protocol. Immediately following the surgery a train of 1 Hz stimulation at 3 V, pulse duration 0.2ms, was administered for 10 seconds. This provided a baseline for comparison to the experimental days.

Based on the 1-day experiments in section 4.4, it was known that there would be a decrease in M-wave amplitude and area and an increase in M-wave duration throughout the experiment. Thus, only the initial cycle of each experimental day was compared to the brief stimulus on the surgery day. There was a significant difference in the M-wave signal when comparing the surgery day and each of the experimental days.

The amplitude and area of each of the experimental days decreased significantly compared to the surgery day, while duration was significantly increased. There were no differences between each of the experimental days. Thus, the decrement of the amplitude and area signals and increases in duration observed at the beginning of the first experimental day was maintained for the beginning of days 2 and 3.

In some cases, the M-wave signal was of low amplitude at the beginning of each experimental day, making it difficult to analyze the data. Qualitatively, in some rats, the shape of the M-wave was different compared to the surgery day. The only difference in timing between the 1 day and 3 day experiments was the 5 days between surgery and the start of the experiment for the 3-day rats. The 1-day rats had the surgery performed the day of the experiment and both groups were sacrificed 3 days following the beginning the experimental protocol. It was also observed that on the day the tissue was harvested, there was a substantial amount of new tissue growth surrounding and adhering the electrode, whereas in the 1-day experiments this growth was not present.

A possible explanation for the change in M-wave signal may have been due to movement of the stimulating and/or recording electrodes. Moving the electrodes would affect the signal as the distance between the electrodes and the orientation to the muscle fibers may change. The electrical stimulation signal as read by the recording electrodes would than have a different shape. However, this does not seem likely as the electrode knots were in the same positions upon harvesting of the tissues. There was a knot located adjacent to

the muscle surface and another against the skin surface for each electrode. As the tissue was being harvested, these knots remained in these locations and there was little slack between them. Also, the electrode implantation procedure was employed in order to maintain electrode stability. The alternative would have been to insert an electrode temporarily for each experimental day. This method was not chosen as it would have been difficult to place the electrode in the same location each day. Also, by implanting electrodes several times, there would be increased likelihood of causing multiple muscle injuries due to multiple needle insertions.

A more plausible explanation for the decrement in the M-wave signal is muscle tissue injury and healing after electrode implantation and/or granulation tissue growth around the electrodes. Increased tissue growth around the electrode could result in a reduced M-wave signal being recorded. Additionally, there were differences in the histology evaluation between the 1-day and 3 day experimental groups, which will be discussed in chapter 5.

Since there was a decrement in the M-wave signal between the surgery day and beginning of each experimental day, a comparison of the raw M-wave data between days would not be reliable. However, a comparison between the beginning and end of each experimental day was performed to assess fatigue within each day. The initial cycle of each day served as the baseline and the last cycle of each day was calculated as a percentage of the initial cycle. This percentage change in M-wave signal was then compared between days.

4.6 Muscle Fatigue Due to Electrical Stimulation with Varied Duty Cycles and Cycle Times – 3 Day Experiments

4.6.1 Specific Aim IV

Since the previous study (section 4.5) determined that the electrode configuration implanted over several days resulted in an unstable M-wave, a comparison of the M-wave signal between days was feasible only by calculating the percentage change from the beginning to the end of each experimental day and comparing these percentages. Also, a comparison of the M-wave signal from the beginning to the end of each day would provide acceptable information on muscle fatigue due to electrical stimulation within an experimental day. Thus, the purpose of this study was to determine the effects of the various stimulation protocols utilized in section 4.4 on the M-wave measurements for 2 hours for each of 3 consecutive days.

4.6.2 Statistical Analysis

Data was recorded over a 3-day period for 2 hours each day. Since there was a significant difference between the surgery day and the beginning of each day, the M-wave measurements of amplitude, area, and duration were converted into percentages of the initial cycle for that day. Specifically, the initial cycle and the final cycle (2 hours) were analyzed for each day with each 2-hour cycle converted into a percentage of the initial cycle.

The analyses were performed on 24 Male Sprague-Dawley rats (400-450g). At the beginning of each day's final 2 hour cycles, for 10 waveforms the mean and standard error was calculated for the percentage of the initial cycle M-wave measurements from the 24 rats. The nonparametric Wilcoxin test was

performed to identify which point(s) showed the greatest changes from the day's initial cycle.

The rats were divided into their respective work/rest group (table 4-1) and Wilcoxin tests were performed to determine the cycles that differed for the groups each day and between days. A comparison between each group using the Mann-Whitney Test was then performed. The groups were separated into duty cycle and cycle time groups and the Wilcoxin and Mann-Whitney tests were repeated on these groups.

In order to determine fatigue within a cycle, the beginning and end of the final 2-hour cycle for days 1, 2, and 3 were averaged. Therefore, for each cycle analyzed, there was an average M-wave analysis at the beginning and end, similar to the 1-day experiment. The percent difference was then calculated between the initial and final measurements of each cycle and averaged. Wilcoxin tests were performed comparing these beginning and end percent differences of each cycle to determine if there was a significant change in the M-wave signal within each cycle. Also, Mann-Whitney tests were performed on the differences between the beginning and end of the cycle comparing each group, as well as the duty cycles and cycle time groups.

An alpha level of $p<0.05$ was considered significant for these analyses. All statistical procedures were performed in PASW Statistics v18.1 (SPSS, Inc Chicago, IL). The nonparametric tests were used due to non-normal distribution.

4.6.3 Results

The pooled data (n=24) indicated that the M-wave amplitude at day 1 and day 3 decreased significantly from the beginning of the day's experiment (figure

4-25). The day 2 cycle showed an increase in amplitude that was not significant. M-wave area remained constant at each cycle on a given day and did not significantly differ from the beginning of that day. M-wave duration increased above 100% at each cycle and significantly at day 1 and 3.

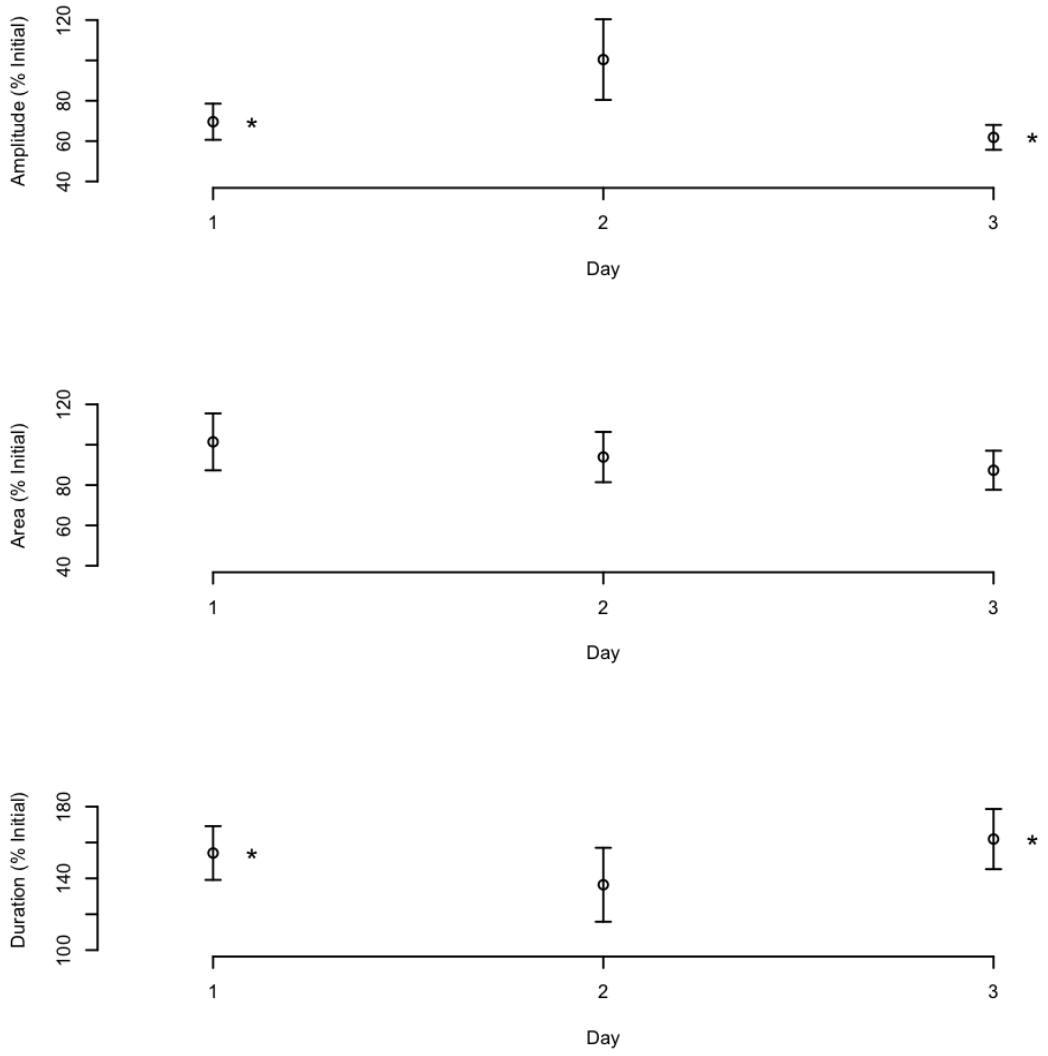


Figure 4-25: Overall average values of M-wave amplitude, area, and duration comparing each of the specified cycles throughout the 3-day experiment. Significant differences ($p<0.05$) comparing the beginning to the end of each day are indicated by the * symbol.

4.6.3.1 Work/Rest Group Comparison Between Cycles

The overall results were broken down into the work/rest groups. In general, the M-wave amplitude decreased at each cycle, except the day 2 cycle for groups B and D (figure 4-26). There were significant decreases at day 1 for group A. Group B also had a significant difference at the day 1 cycle, as well as day 3. Group C resulted in the least variation and had significant differences at days 2 and 3. The group D amplitude decreased significantly at day 3. A comparison between days revealed a significant difference at day 2 for group B compared to days 1 and 3.

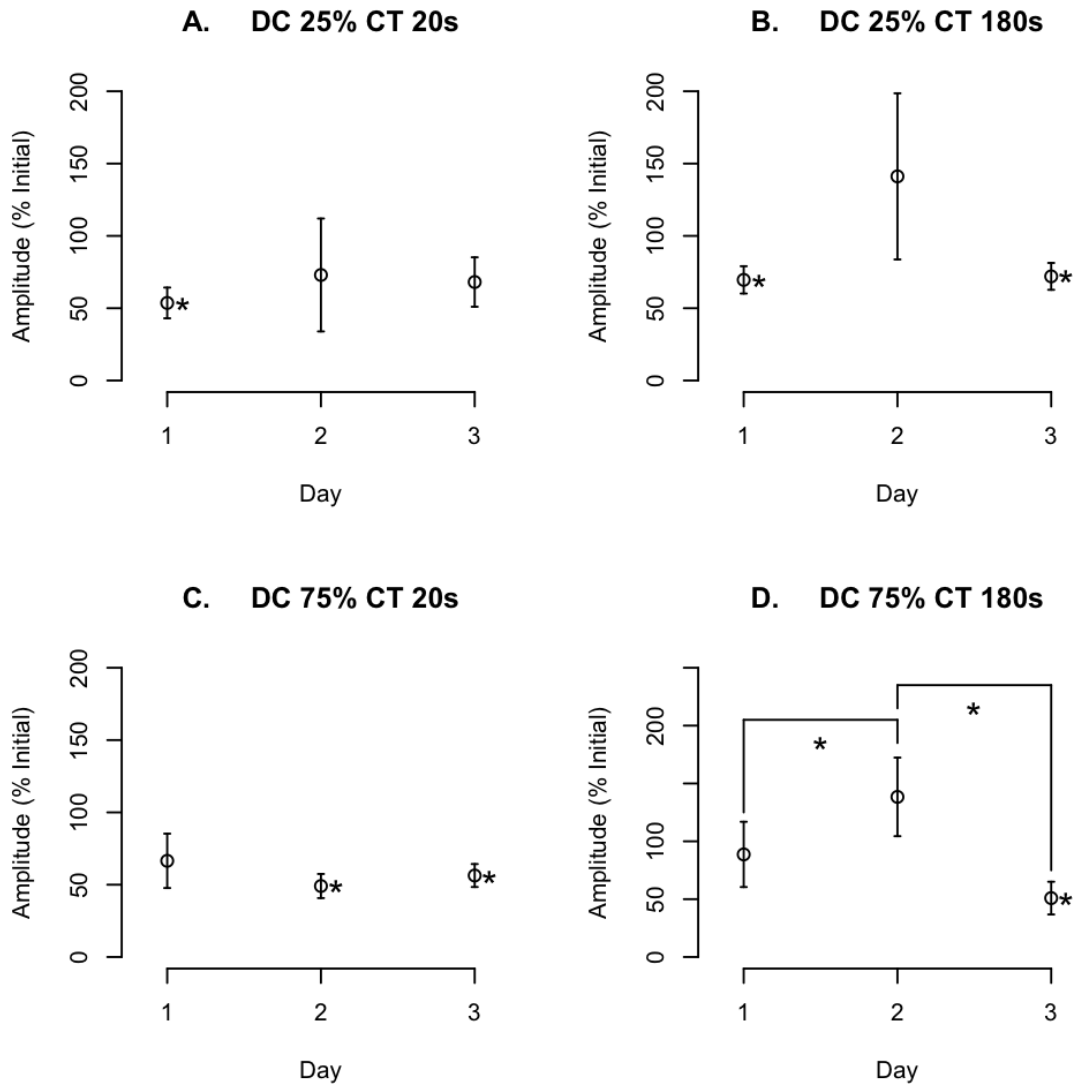


Figure 4-26: Average M-wave amplitude values comparison of the specified cycles throughout the 3-day experiment for each work/rest group. Significant differences ($p<0.05$) comparing the beginning to the end of each day are indicated by the * symbol. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

The work/rest groups for M-wave area remained similar to the initial cycle for each day (figure 4-27). Only the day 3 cycle for group C had a significant decrease in M-wave area from the initial cycle. This percent decrease was also significantly different from the M-wave area measured at day 1. In group D, the area at day 3 was significantly different than at day 2.

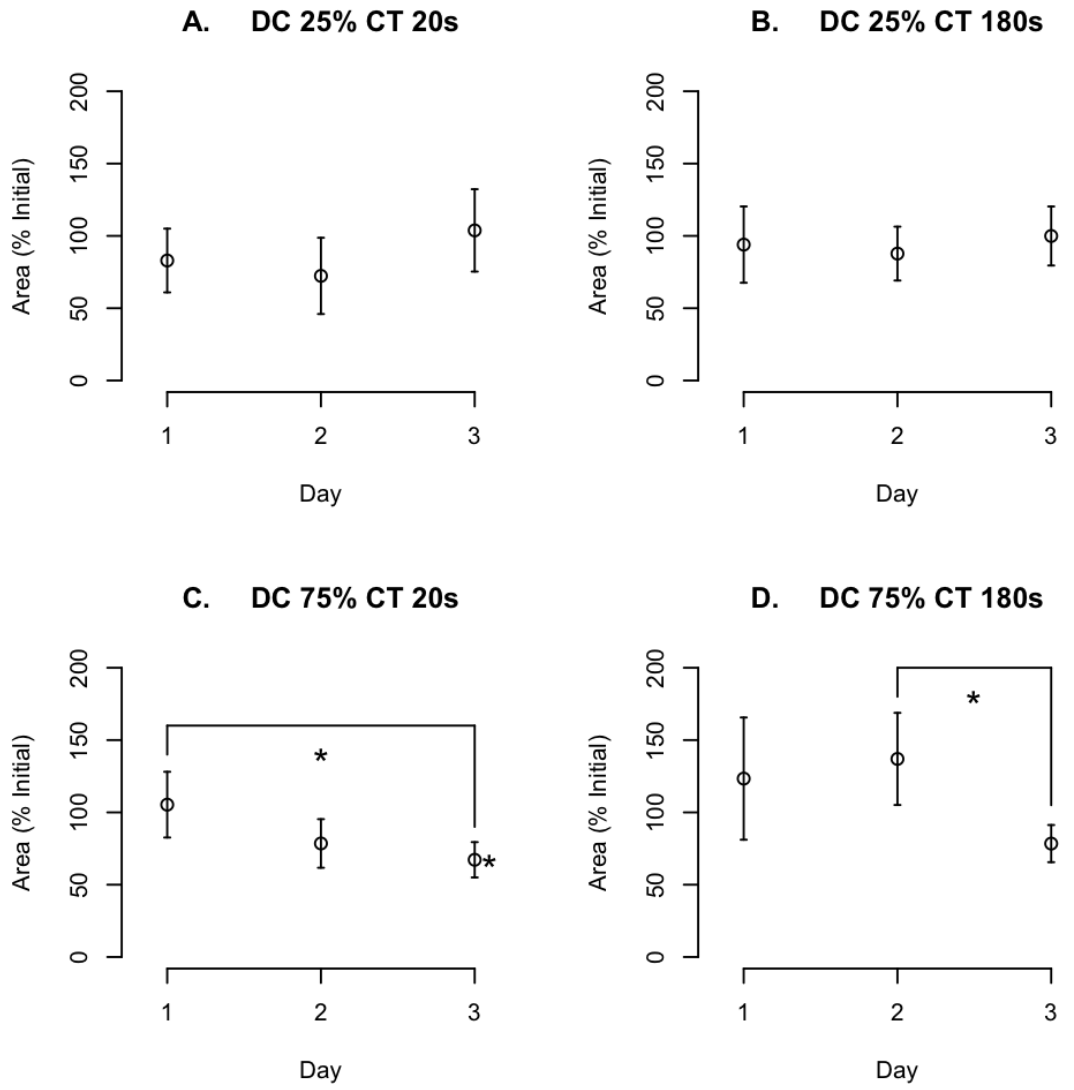


Figure 4-27: Average M-wave area values comparison of the specified cycles throughout the 3-day experiment for each work/rest group. Significant differences ($p<0.05$) comparing the beginning to the end of each day are indicated by the * symbol. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

On day 1, groups A, B, and D had significant increases in M-wave duration percentage, while group C was not significantly elevated (figure 4-28). Day 2 saw no significant increases in any of the groups. Groups A and D were significantly elevated at day 3 compared to their initial cycles for that day. In

group A, the increases in percent M-wave duration were significantly higher at days 1 and 3 compared to day 2.

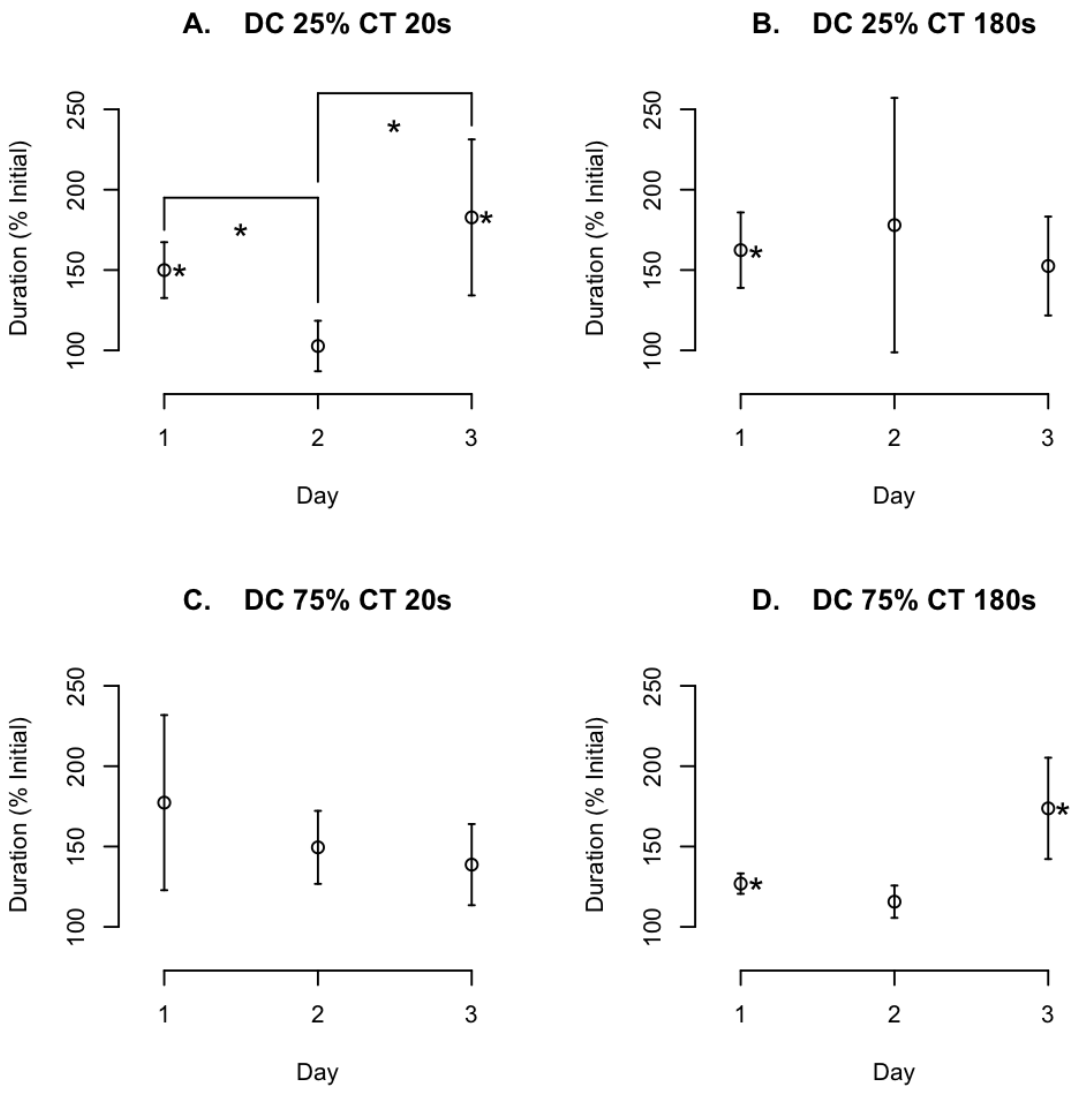


Figure 4-28: Average M-wave duration values comparison of the specified cycles throughout the 3-day experiment for each work/rest group. Significant differences ($p<0.05$) comparing the beginning to the end of each day are indicated by the * symbol. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

A comparison between groups found there were no M-wave amplitude differences between any of the 4 groups at day 1 (figure 4-29). At day 2, group C had significantly higher amplitude compared to group D. There were no

significant differences between any of the groups on day 3. Comparing the M-wave area between the groups showed no significant differences. Also, there were no significant differences between the groups for the percent increases in M-wave duration.

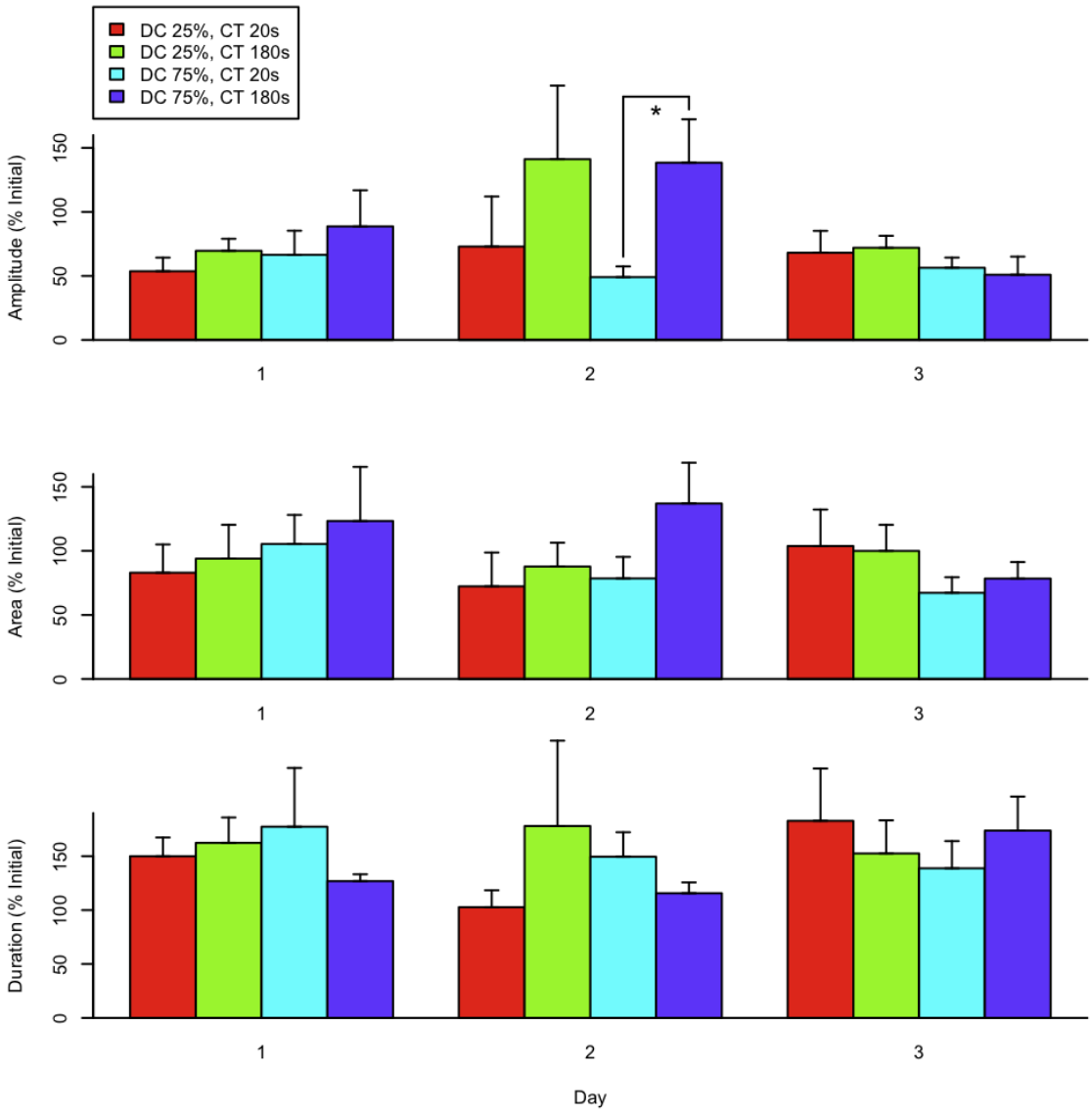


Figure 4-29: Average M-wave amplitude, area, and duration values comparison of each of the work/rest groups at the specified cycles throughout the 3-day experiment. Significant differences ($p<0.05$) are indicated by brackets and the * symbol.

4.6.3.2 Duty Cycle and Cycle Time Comparison Between Cycles

There were decreases in M-wave amplitude for both 25% and 75% duty cycles at day 1 and 3, but not at day 2 (figure 4-30). These decreases were significant except the day 1 cycle for the 75% duty cycle. Cycle time for 20s decreased significantly for each of the 3 cycles. For the 180s cycle time only the day 2 cycle did not significantly decrease, resulting in significant differences between the amplitudes measured at day 1 and 3.

There was less effect of duty cycle and cycle time on the M-wave area compared to the M-wave amplitude (figure 4-31). Only the day 3 cycle for the 75% duty cycle group had a significant decrease in area. This was also significantly different compared to the day 2 percent area. Each of the other cycles for all duty cycle and cycle time groups maintained a constant area.

Duration of the M-wave increased for each cycle for all of the duty cycle and cycle time groups (figure 4-32). At day 1 and 3, this increase was significant for the 25% duty cycle, 20s and 180s cycle time groups. The 75% duty cycle group had significant increases at all 3 cycles. There were no significant differences found between any of the days.

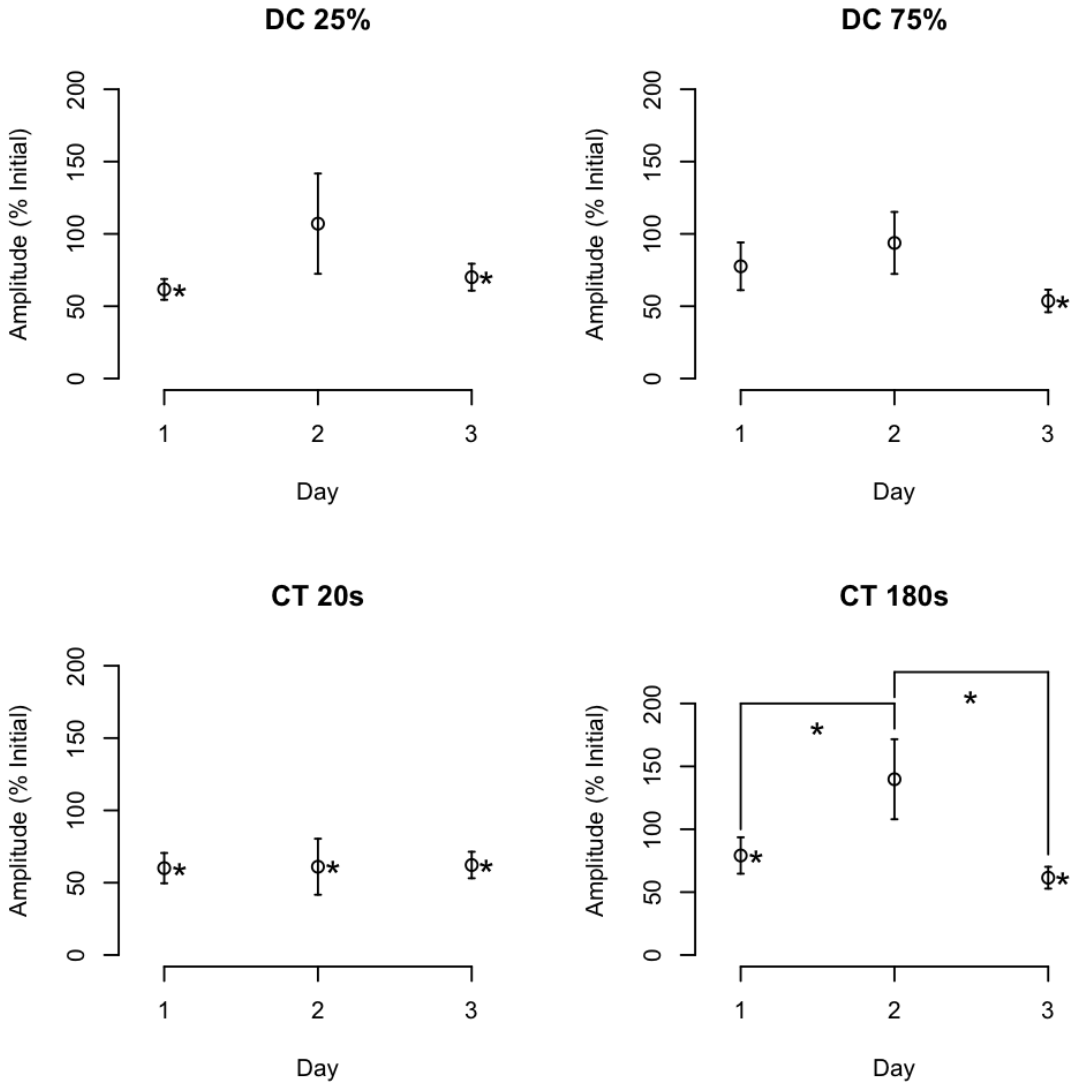


Figure 4-30: Average M-wave amplitude values comparison of the specified cycles throughout the 3-day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) comparing the beginning to the end of each day are indicated by the * symbol. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

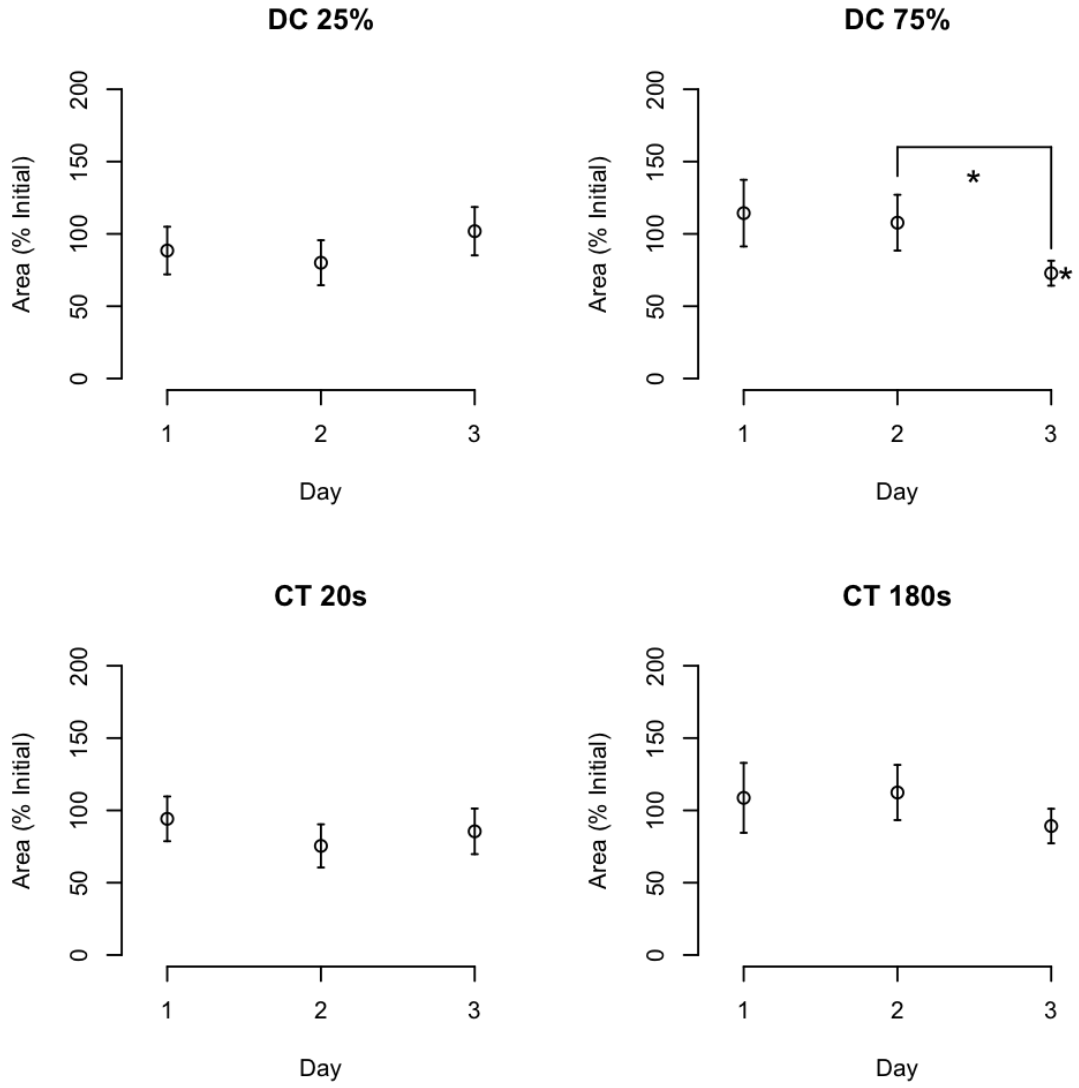


Figure 4-31: Average M-wave area values comparison of the specified cycles throughout the 3-day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) comparing the beginning to the end of each day are indicated by the * symbol. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

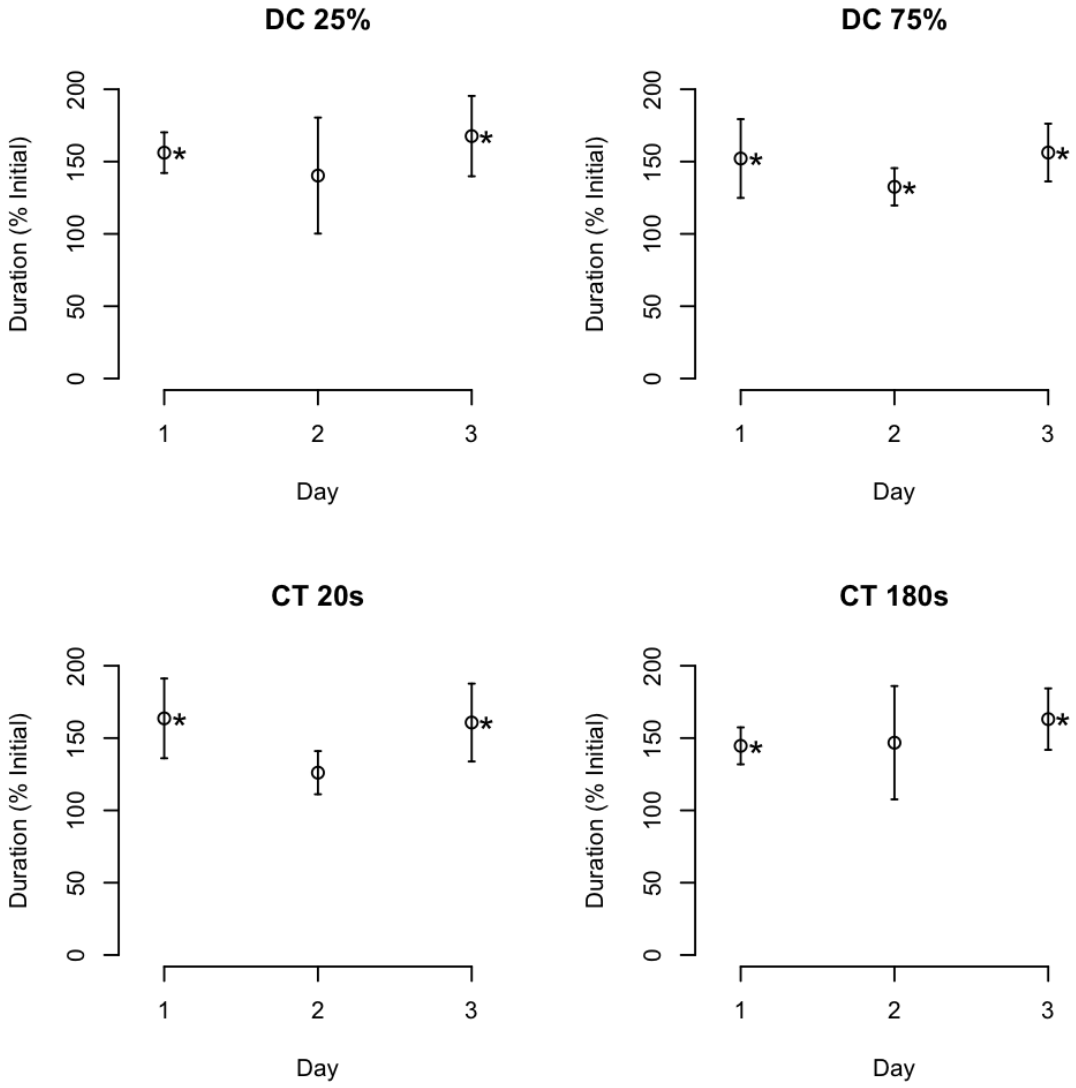


Figure 4-32: Average M-wave duration values comparison of the specified cycles throughout the 3-day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) comparing the beginning to the end of each day are indicated by the * symbol.

The differences between the 25% and 75% duty cycle groups did not result in significant differences at any cycle for each of the measures of M-wave amplitude, area, and duration (figure 4-33). The 25% duty cycle group had greater percent amplitude than the 75% duty cycle group at day 2 and 3 but not at day 1. For area this trend was reversed as only at the day 3 cycle the 25%

duty cycle was larger than the 75% duty cycle. Duration was increased for the 25% duty cycle group at each cycle compared to the 75% duty cycle group.

In the cycle time comparisons, only the M-wave amplitude at day 2 produced a significant difference with the 180s cycle time group having a larger percent amplitude than the 20s cycle time group. The amplitude at day 1 also was increased for the 180s cycle time group, while there was a slightly larger amplitude for the 20s cycle time group at day 3. At each cycle, the 180s cycle time group resulted in a larger M-wave area compared to the 20s cycle time group. Duration of the M-wave was longer at day 2 and 3 for the 180s cycle time group, but shorter than the 20s cycle time group at day 1.

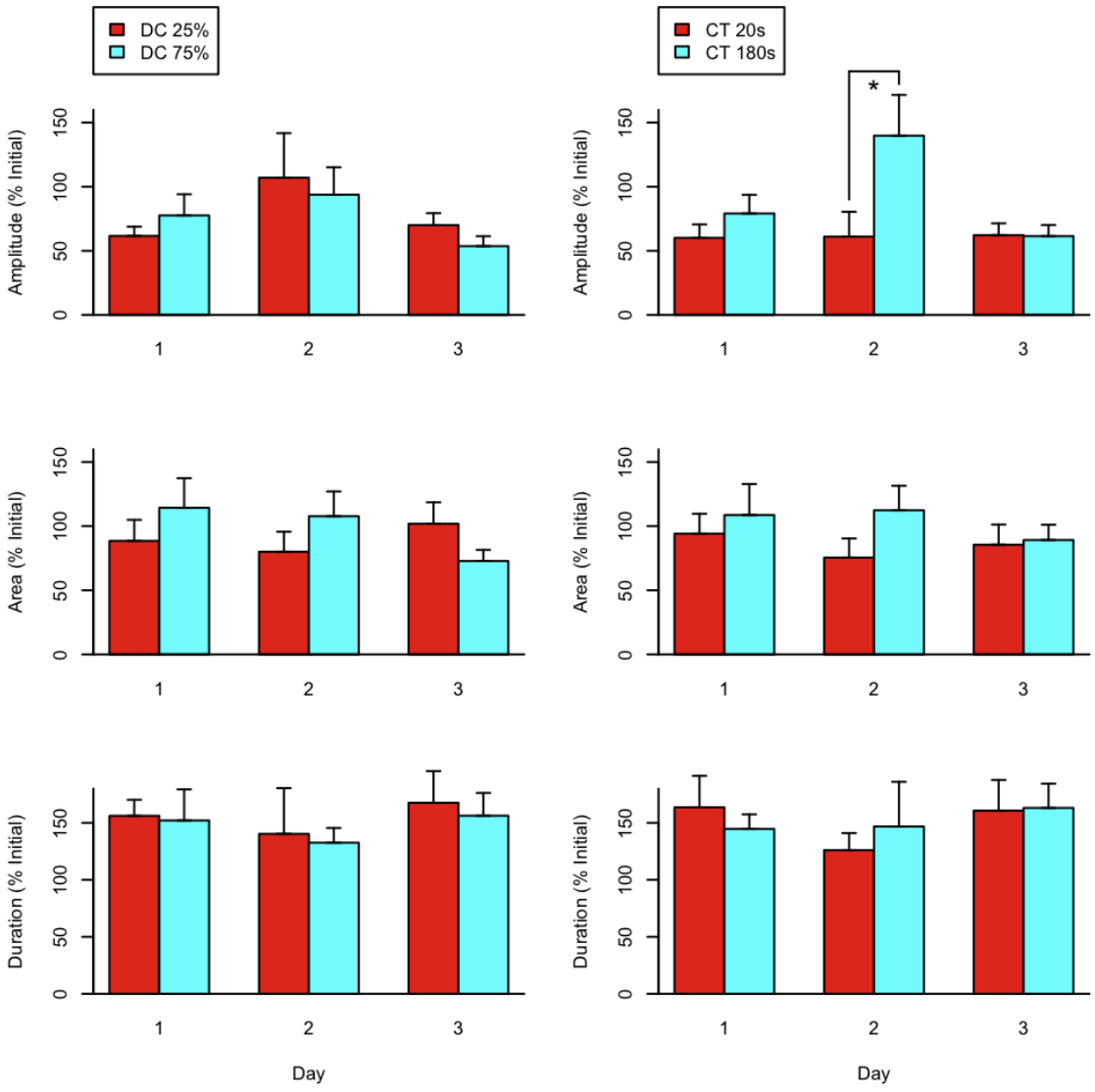


Figure 4-33: Average M-wave amplitude, area, and duration values comparison of each of the duty cycle and cycle time groups at the specified cycles throughout the 3-day experiment. Significant differences ($p<0.05$) are indicated by brackets and the * symbol.

4.6.3.3 Work/Rest Group Comparison From the Beginning to the End of Each Cycle

With the groups pooled (n=24), there was a decrease in M-wave amplitude from the beginning to the end of the cycle for each time point and each of these decreases were significant (figure 4-34). M-wave area also decreased from the beginning to the end of each cycle, with the day 2 and 3 cycles

decreasing significantly. Duration of the M-wave increased from the beginning to the end of each cycle. The day 1 and 2 cycles resulted in significant increases.

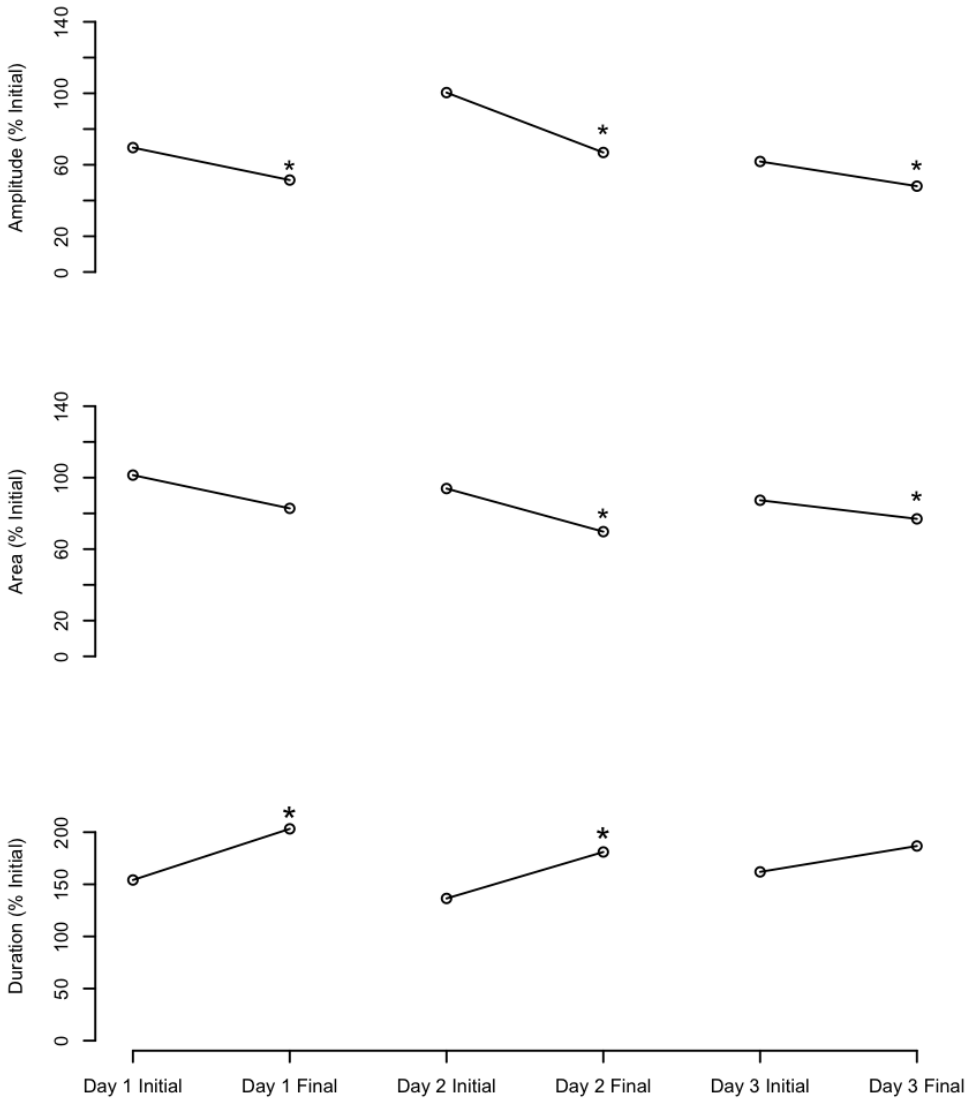


Figure 4-34: Overall average M-wave amplitude, area, and duration comparing the beginning to end of each of the specified cycles throughout the 3 day experiment. Significant differences ($p<0.05$) comparing the beginning to the end of each day are indicated by the * symbol.

The analysis of each group separately showed a decrease in percent amplitude of the M-wave from the beginning to the end of each cycle for every group (figure 4-35). Group A had the least change in amplitude, resulting in no

significant decreases. Although the decrease in amplitude for group C averaged approximately 20%, the 4 hour and 6 hour cycles were significant. The day 2 cycle for groups B and D had the largest amplitude changes, resulting in significant decreases, which may have been attributable to the large initial amplitudes. The decrease in amplitude at day 3 for group D was also significant.

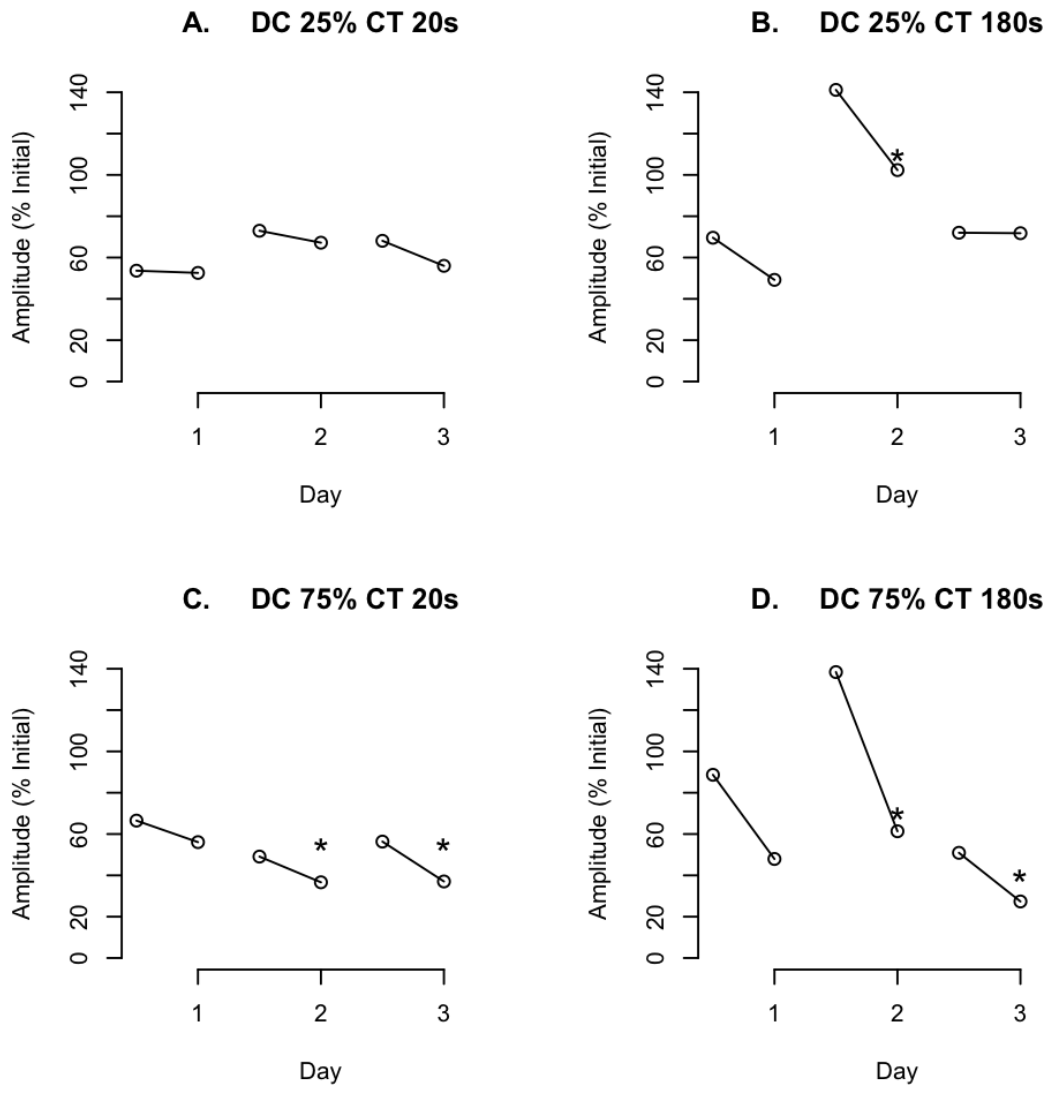


Figure 4-35: Average M-wave amplitude comparison from the beginning to end of each of the specified cycles throughout the 3 day experiment for each work/rest group. Significant differences ($p<0.05$) are indicated by the * symbol.

Although the pooled area data resulted in decreases in M-wave area from the beginning to the end of each cycle, the individual group data showed some increase in M-wave area (figure 4-36). Group A area increased from the beginning to the end of each cycle, but these increases were not significant. Group B also had an increase in area at day 3, with decreasing areas at day 1 and 2. None of those changes were significant. The M-wave area for each cycle for groups C and D decreased from the beginning to the end of the cycles. The day 2 and 3 cycle decreases were significant for both groups.

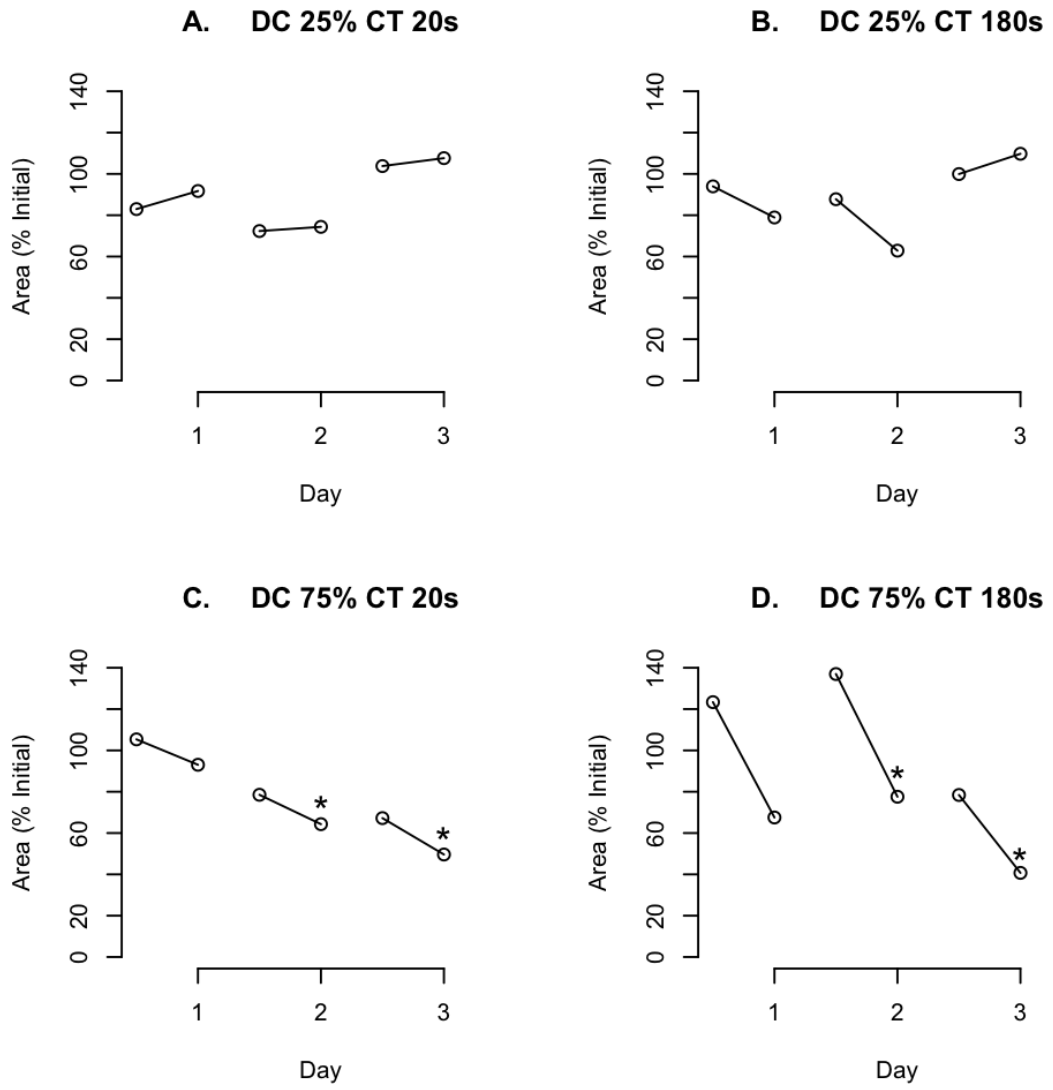


Figure 4-36: Average M-wave area comparison from the beginning to end of each of the specified cycles throughout the 3 day experiment for each work/rest group. Significant differences ($p<0.05$) are indicated by the * symbol.

M-wave duration for all the groups increased from the beginning to the end of each of the cycles (figure 4-37). The increases at day 1 for groups A, C, and D were significant, while group B had a significant increase at day 2. Group B had the largest percent increase in M-wave duration.

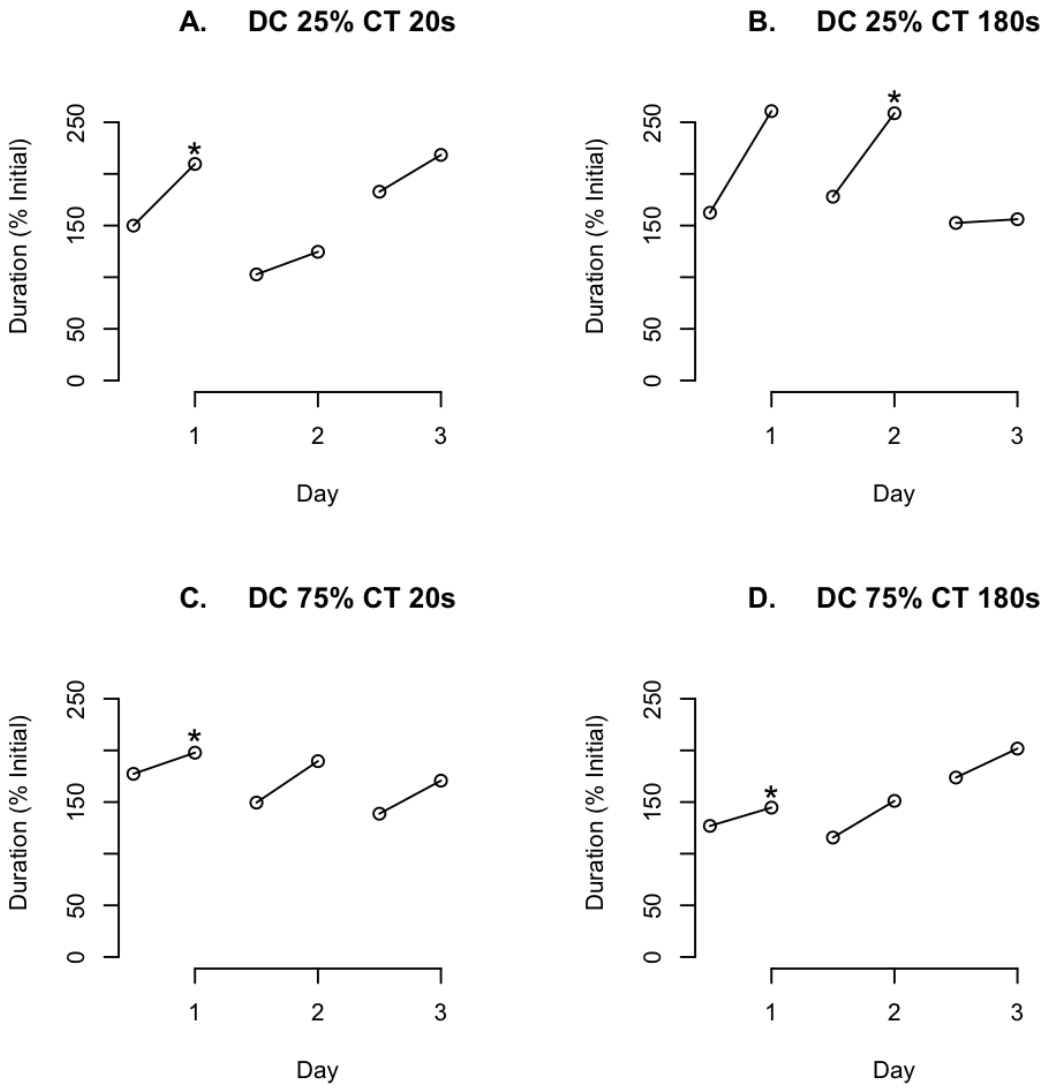


Figure 4-37: Average M-wave duration comparison from the beginning to end of each of the specified cycles throughout the 3 day experiment for each work/rest group. Significant differences ($p<0.05$) are indicated by the * symbol.

The decrease in percent amplitude was greatest for group D and this was significantly different than group A at day 1 and group B at day 3 (figure 4-38). Group D also had the largest decrease in M-wave area and this was significantly different from group B at day 3. There were no significant differences in duration between groups, although group B had the largest changes at day 1 and 2.

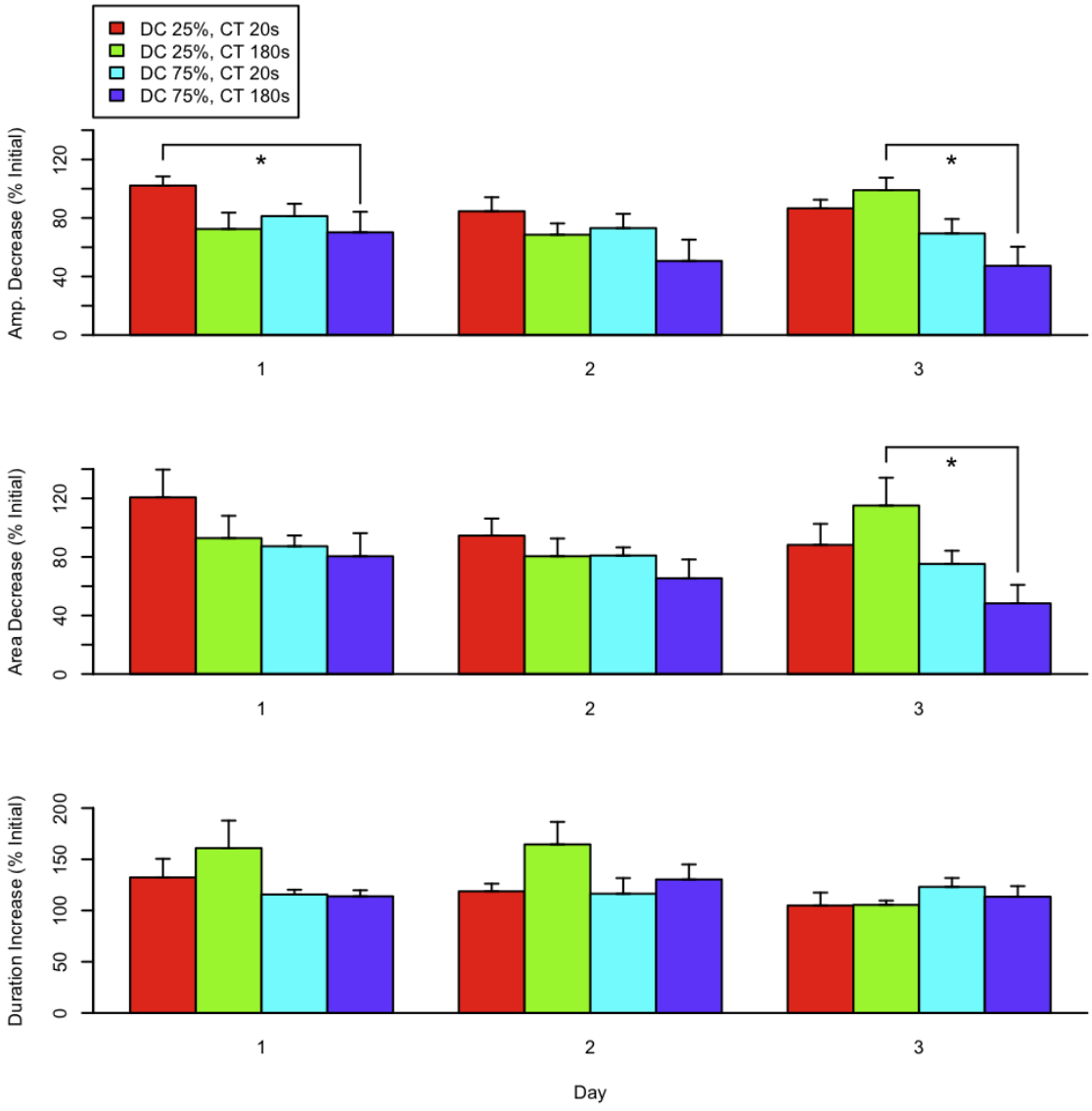


Figure 4-38: Comparison of each of the work/rest group average M-wave amplitude, area, and duration difference from the beginning to the end of each of the specified cycles throughout the 3-day experiment. Significant differences ($p<0.05$) are indicated by brackets and the * symbol.

4.6.3.4 Duty Cycle and Cycle Time Comparison From the Beginning to the End of Each Cycle

The percent decreases in amplitude from the beginning to the end of each cycle were significant for the 75% duty cycle and 180s cycle time groups (figure 4-39). These groups also had the largest decreases in amplitude. The 20s cycle

time group also resulted in significant decreases at day 2 and 3, while the day 2 cycle was significant for the 25% duty cycle group.

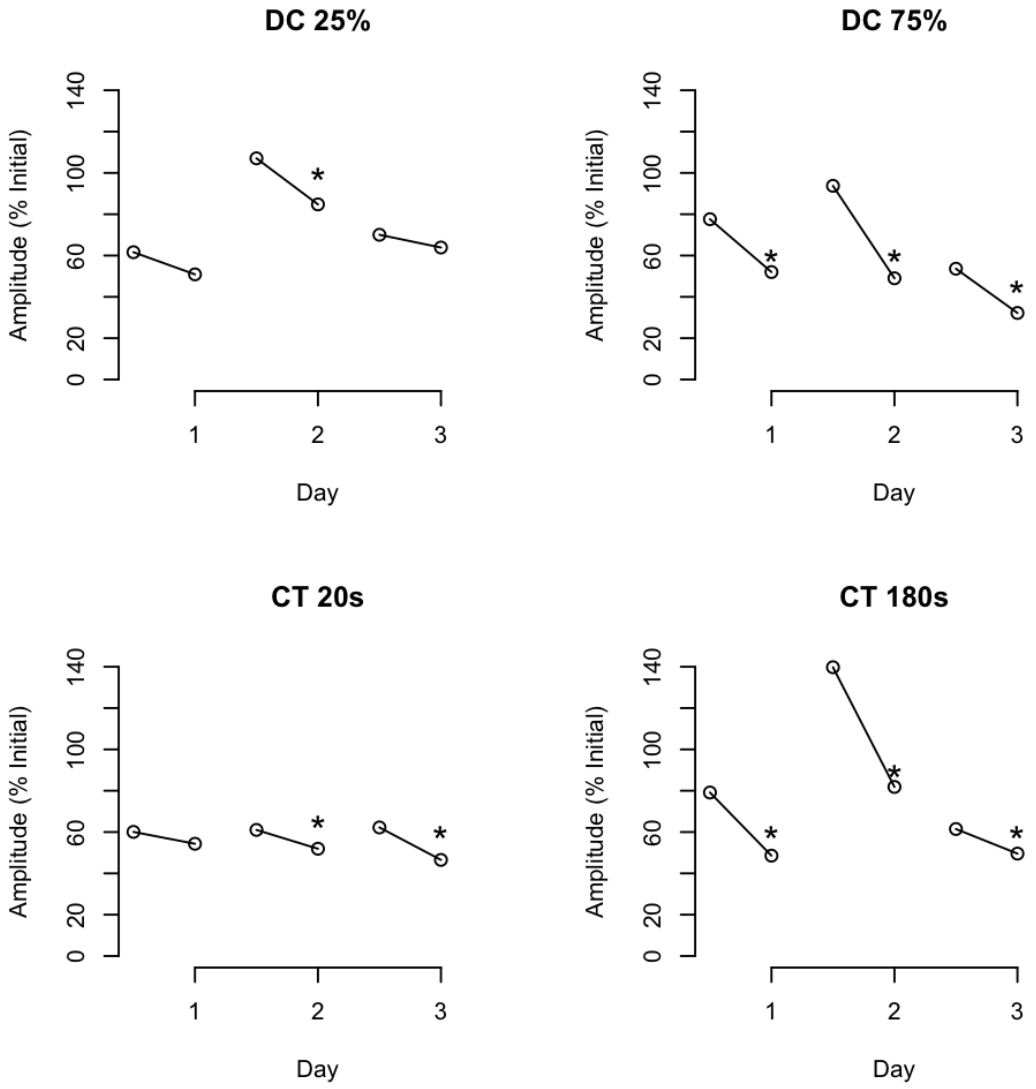


Figure 4-39: Average M-wave amplitude comparison from the beginning to end of each of the specified cycles throughout the 3 day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) are indicated by the * symbol.

The 75% duty cycle and 180s cycle time groups also had the largest decreases in M-wave area from the beginning to the end of each cycle (figure 4-

40). The changes for the 75% duty cycle group was significant for each cycle, while only the day 2 cycle was significant for the 180s cycle time group. There were no significant decreases in area for the 25% duty cycle group and there was an increase in area at the day 3 cycle. There were small decreases in area for each cycle in the 20s cycle time group and the percent change at day 3 were significant.

A percent increase in M-wave duration was observed from the beginning to the end of each cycle for both duty cycle and cycle time groups (figure 4-41). The increases at day 1 and 2 were significant in the 25% duty cycle and 180s cycle time groups. The 75% duty cycle group had significant increases in duration at day 1 and 3. Only the day 1 cycle for the 20s cycle time group resulted in a significant percent increase in M-wave duration.

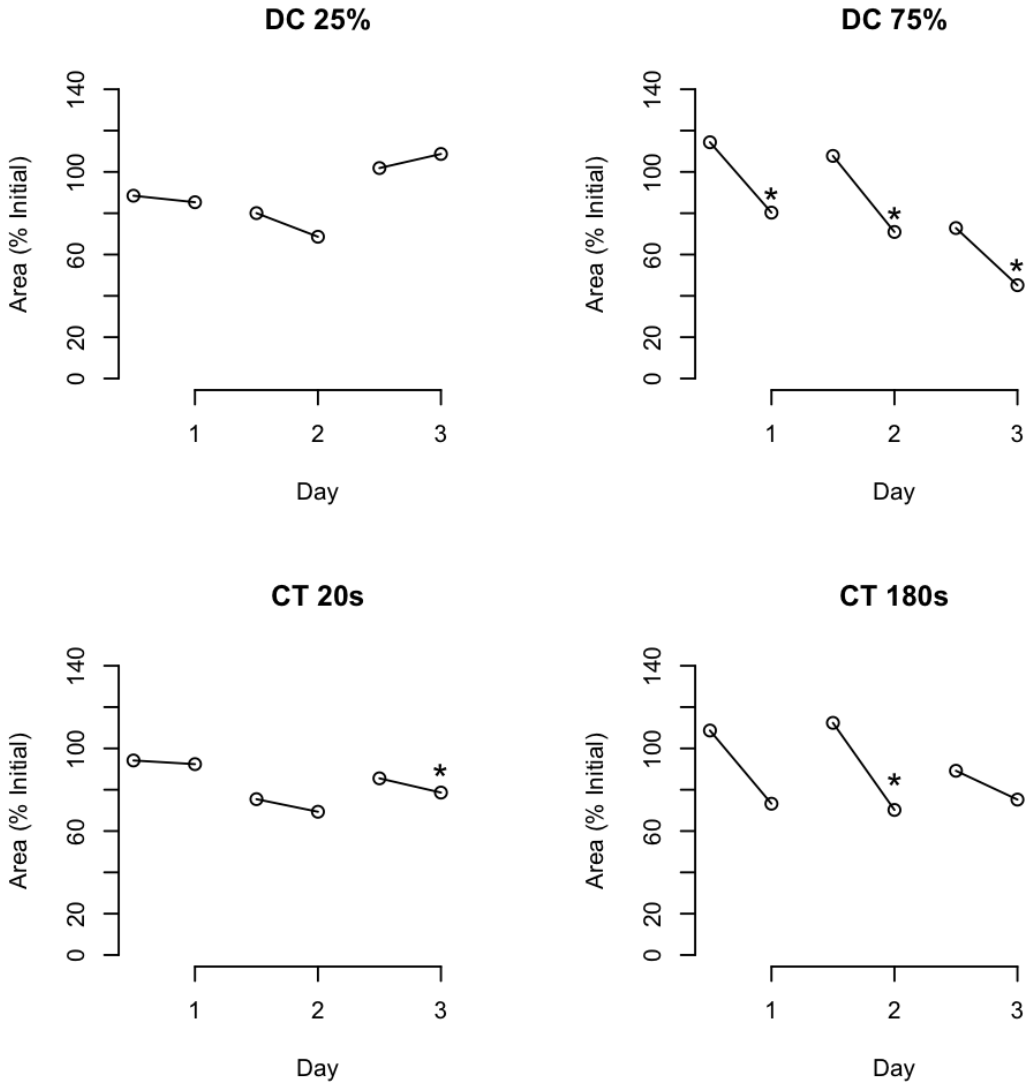


Figure 4-40: Average M-wave area comparison from the beginning to end of each of the specified cycles throughout the 3 day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) are indicated by the * symbol.

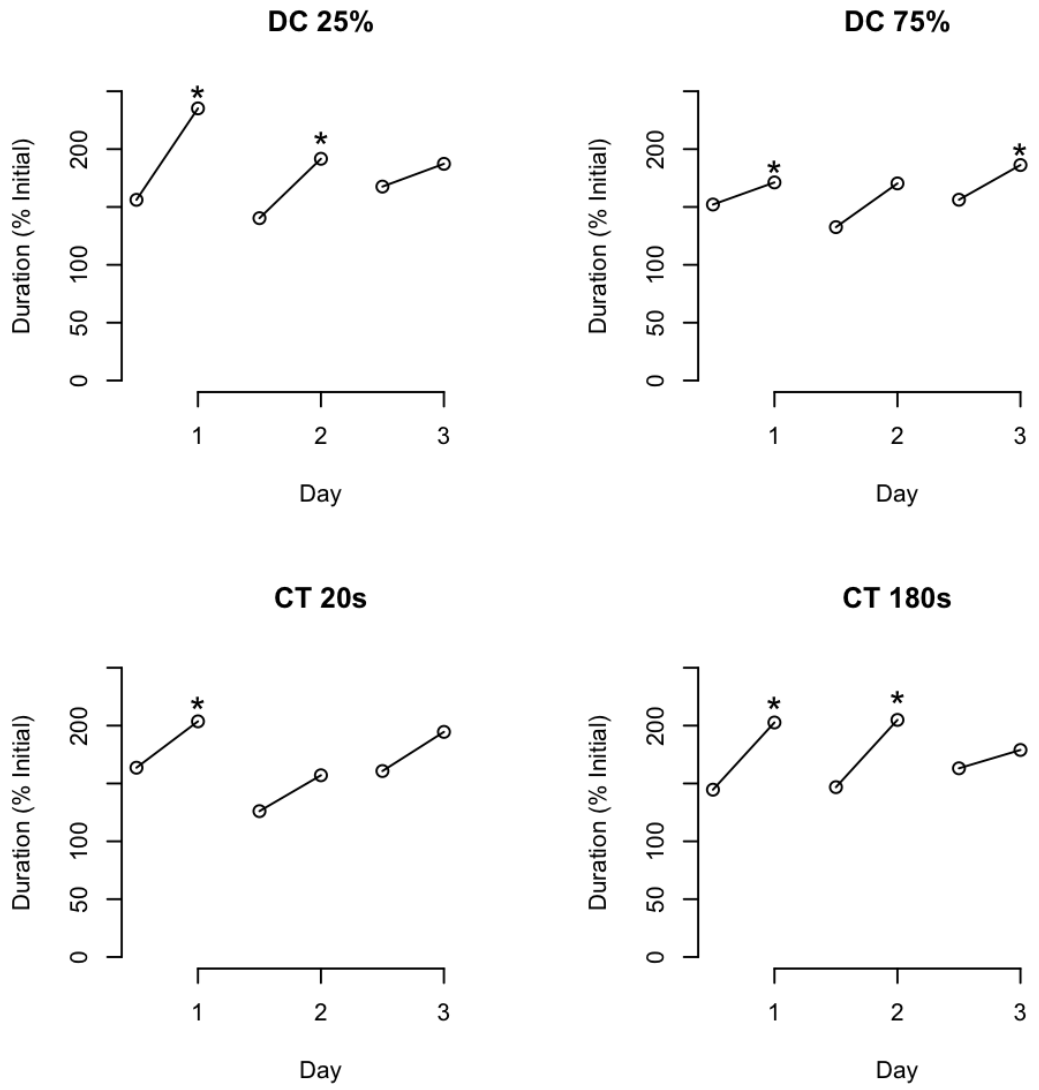


Figure 4-41: Average M-wave duration comparison from the beginning to end of each of the specified cycles throughout the 3 day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) are indicated by the * symbol.

Although there were changes in the M-wave amplitude, area, and duration of cycles in the duty cycle and cycle time groups, the comparison between groups only resulted in 2 significant differences (figure 4-42). Both of the differences occurred at day 3 for the amplitude and area changes between the 25% and 75% duty cycle groups. The 75% duty cycle and 180s cycle time

groups had greater percent decreases in amplitude and area between the beginning and end of each cycle. The M-wave duration increase was longer at day 1 and 2 for the 25% duty cycle and 180s cycle time groups.

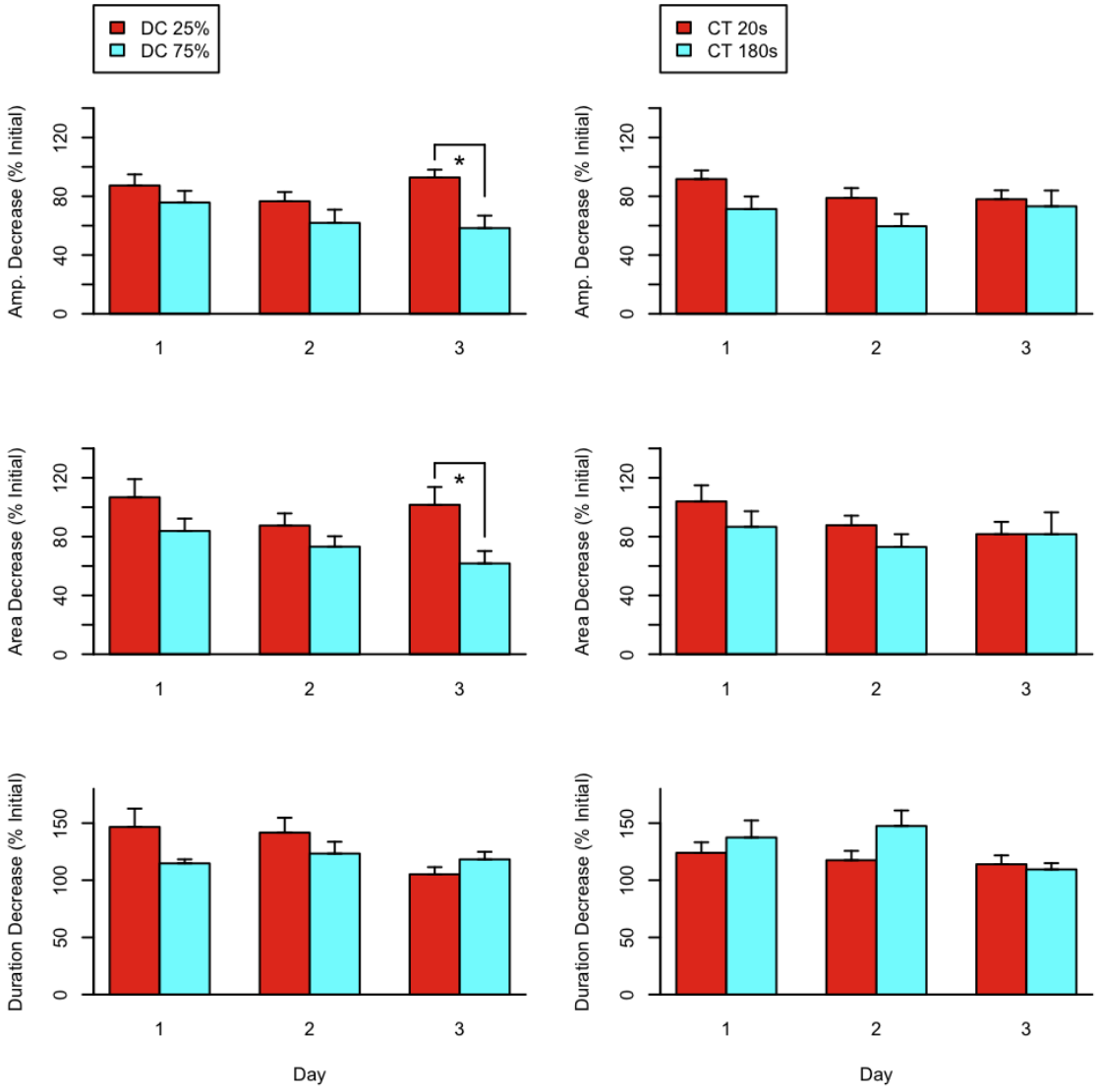


Figure 4-42: Comparison of each of the duty cycle and cycle time group average M-wave amplitude, area, and duration difference from the beginning to the end of each of the specified cycles throughout the 3-day experiment. Significant differences ($p<0.05$) are indicated by brackets and the * symbol.

4.6.4 Discussion

The physiological indicators of fatigue were present in the 3-day experiment. On days 1 and 3, the M-wave amplitude from the beginning to the end of the 2-hour stimulation decreased and M-wave duration increased significantly. The variability was large on day 2 and may have contributed to the non-significant changes in amplitude and duration. Area of the M-wave did not decrease significantly each of the 3 days. This may have been due to the small signal strength, discussed in section 4.5.

A comparison between experimental days did not reveal a difference based on days. Each group had similar percent changes in amplitude, area, and duration measurements at the end of each day. There were only a few examples in which the measurements between days differed significantly. These differences were questionable due to large variability and the difficulty in measuring M-wave attributes because of the small signals. Thus, an influence of experimental day on fatigue within each group was not supported. Similarly, experimental day did not influence fatigue due to duty cycle or cycle time.

A comparison between groups at the specific time points also revealed little differences. Only the amplitude comparison between groups B and D at the day 2 cycle resulted in a significant difference with group C having smaller M-wave amplitude. It can be suggested that cycle time was the reason for this difference as the amplitude for the 20s cycle time was significantly different than the 180s cycle time at day 2. However, since this was the only significant difference, it did not allow for a strong conclusion that cycle time, particularly in groups C and D were significantly different. Therefore, although M-wave

amplitude and area generally decreased and duration increased each day, the differences between work/rest groups, duty cycle, and cycle time does not allow for a differentiation based on statistical significance between these fatigue measures.

Comparing the changes within each day 1 cycle provided expected results. Overall, M-wave amplitude and area decreased, while duration increased from the beginning to the end of each cycle. This was also seen in each experimental group and each duty cycle and cycle time groups. Group D had the largest changes in M-wave amplitude and area, resulting in significant differences compared to other groups.

Duty cycle may have had a cumulative effect on fatigue from the beginning to the end of each cycle by the 3rd day. There was a general trend of decreasing amplitude and area in the 75% duty cycle group. On day 3 there were significant differences between the 25% and 75% duty cycle groups for both amplitude and area. However, there was also an increase in the amplitude and area of the 25% duty cycle group on day 3, which may have also contributed to the significant difference.

4.7 Muscle Fatigue Due to Electrical Stimulation with Varied Duty Cycles and Cycle Times – 1 and 3 Day Comparison

4.7.1 Specific Aim V

Experiments have been performed to determine the effects of various stimulation protocols over a continuous 6-hour duration (section 4.4) and continuous 2-hour stimulation for each of 3 consecutive days. The percent change in the M-wave signal at the end of each of the 3 days compared to the

beginning of each day was determined. A similar calculation in the M-wave signal can be determined for the 6-hour continuous experiment by taking the percent change at 2 hour increments compared to the initial cycle. Thus, the purpose of this study was to compare the percent change in the M-wave signal between the 1-day and 3 day experiments at specific time points to determine if the number of days of stimulation affects fatigue.

4.7.2 Statistical Analysis

In order to compare the 1-day and 3 day experiments, the 1-day experiment data was converted into percentage of baseline values. The initial cycle was selected as the baseline and the measurements at 2, 4, and 6 hours were converted into percentages of the baseline. The 3-day experiment cycles were relabeled from day 1, 2, and 3 to 2, 4, and 6 hours, to correspond with the 1-day experiment.

The analyses were performed on 52 Male Sprague-Dawley rats (400-450g), with 28 rats coming from the 1-day experiment and 24 from the 3-day experiment. At each of the 3 cycles, percent mean and standard error of the amplitude, area, and duration was calculated. The nonparametric Friedman test was performed to determine any significant differences between the cycles for all the stimulus artifact and M-wave data. Where applicable, the Wilcoxin test was performed to identify which point(s) showed the greatest changes.

In order to determine fatigue within a cycle, in addition to the averaged data obtained at the beginning of each of the 3 cycles, the final 10 waveforms of each of the 3 cycles were averaged. Therefore, for each cycle analyzed, there was an average M-wave analysis at the beginning and end, similar to the 1-day

experiment. The percent difference was then calculated between the initial and final measurements of each cycle and averaged. Mann-Whitney tests were performed between the work/rest groups, and duty cycle and cycle time groups between cycles and from the beginning to the end of each cycle.

An alpha level of $p<0.05$ was considered significant for these analyses. All statistical procedures were performed in PASW Statistics v18.1 (SPSS, Inc Chicago, IL). These nonparametric tests were used due to non-normal distribution.

4.7.3 Results

4.7.3.1 Work/Rest Group Comparison Between Cycles

Overall, there was a larger percent decrease in M-wave amplitude and area and a larger increase in duration at each time point for the 1-day experiment compared to the 3-day experiment (figure 4-43). However, these differences were not significant. Comparing each work/rest group also found amplitude and area with a greater decrease for the 1-day experiment, while duration increased, compared to the 3-day experiment (figure 4-44, 4-45, 4-46). Only the group C area comparison at 2 hours and group A duration comparison at 4 hours resulted in significant differences.

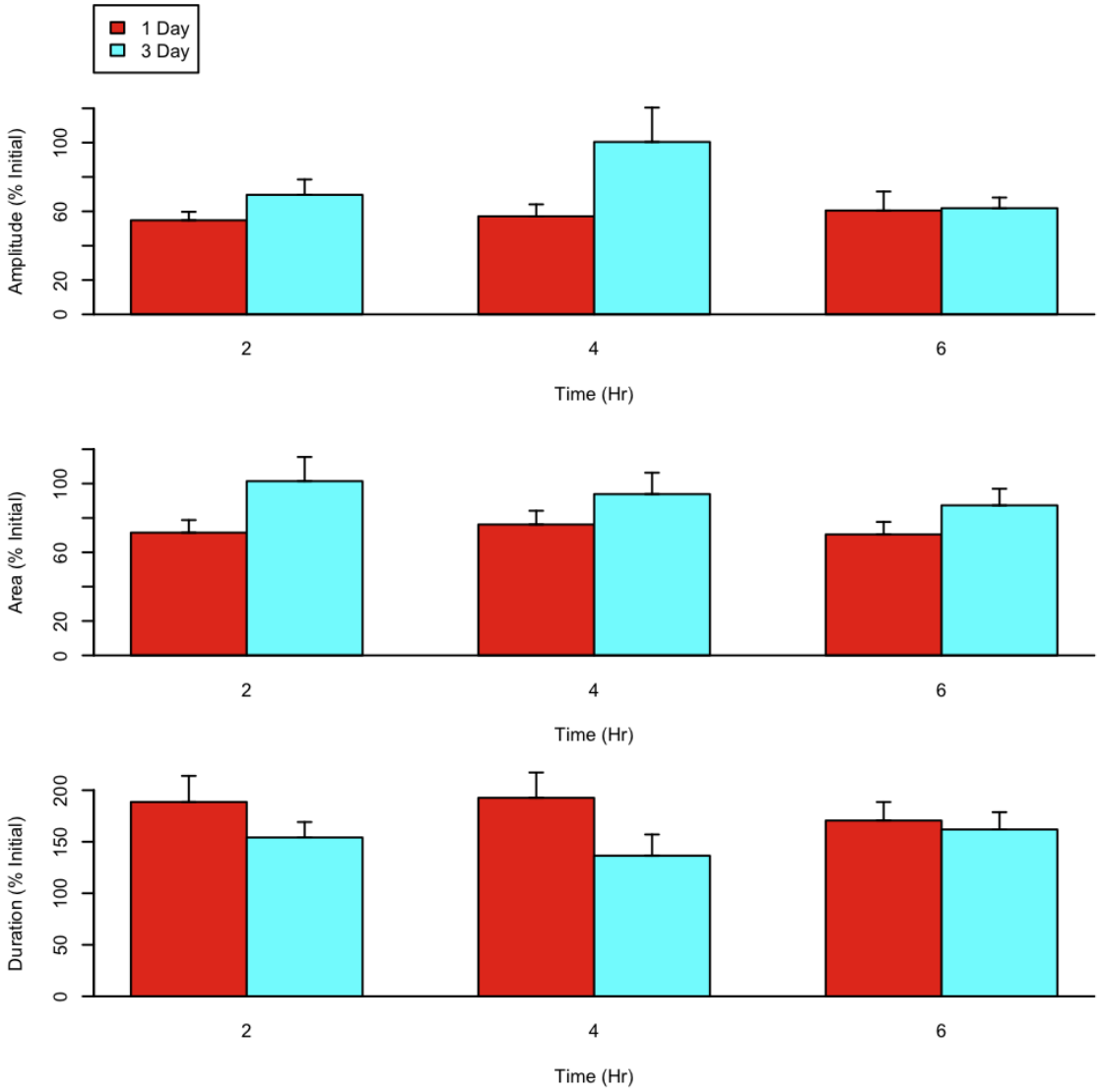


Figure 4-43: Overall average M-wave amplitude, area, and duration comparing each of the specified cycles throughout the 1 and 3 day experiment.

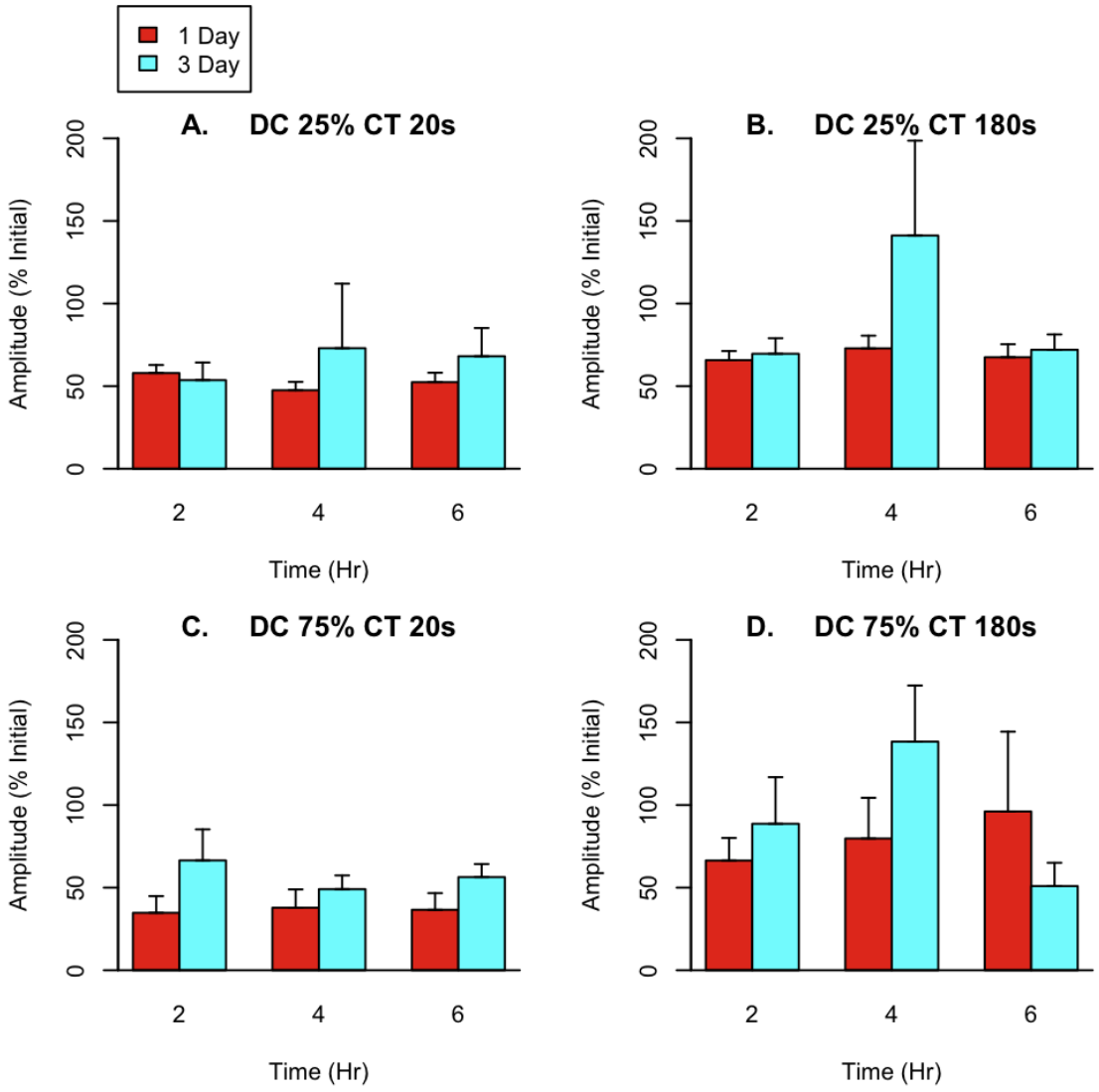


Figure 4-44: Average M-wave amplitude comparison of the specified cycles throughout the 1 and 3 day experiment for each work/rest group.

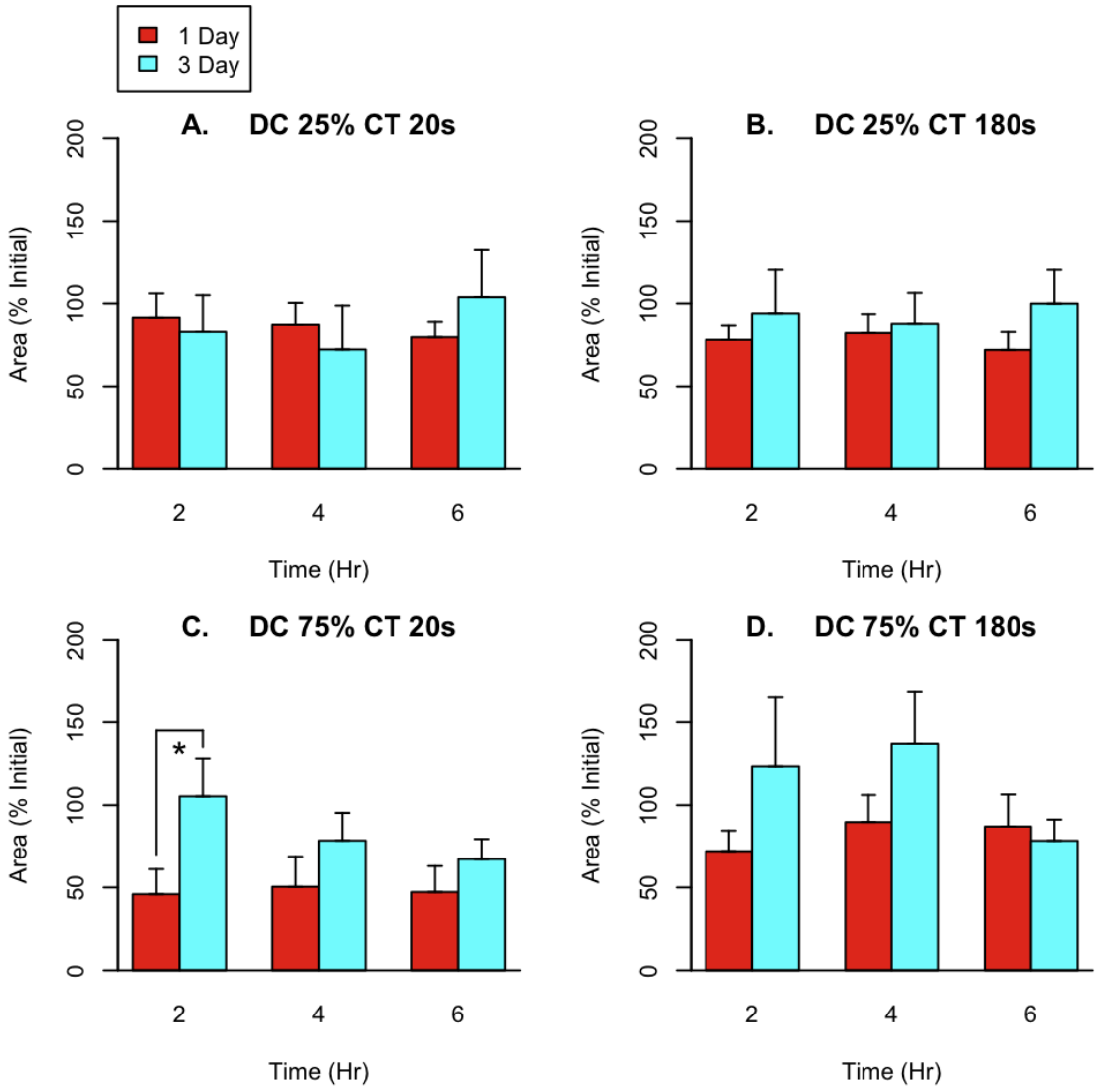


Figure 4-45: Average M-wave area comparison of the specified cycles throughout the 1 and 3 day experiment for each work/rest group. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

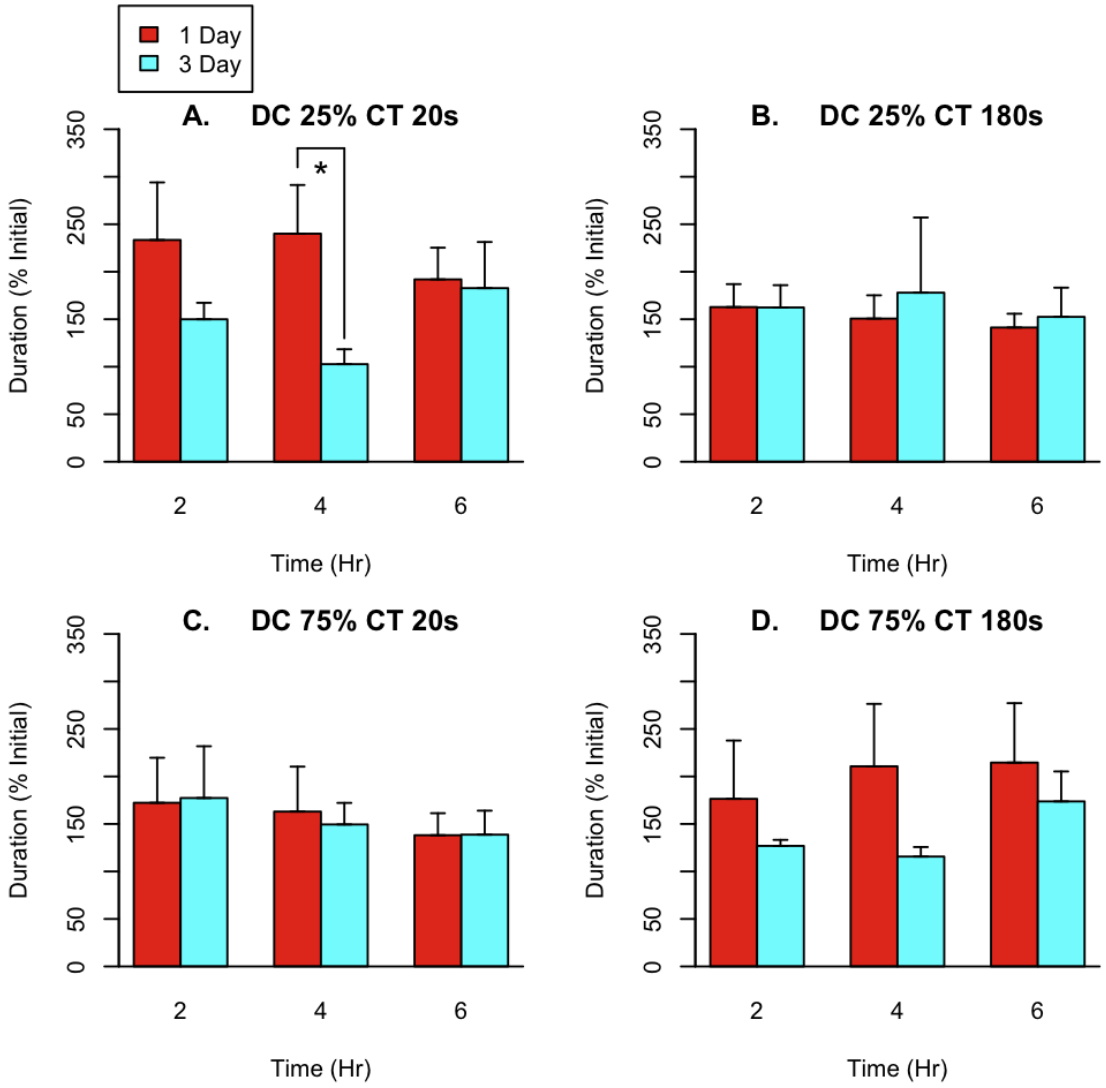


Figure 4-46: Average M-wave duration comparison of the specified cycles throughout the 1 and 3 day experiment for each work/rest group. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

4.7.3.2 Duty Cycle and Cycle Time Comparison Between Cycles

The same trends were generally true when comparing the 1-day and 3 day experiment groups for duty cycle and cycle times, where the 1 day experiment group had greater decreases in amplitude and area and greater increases in duration compared to the 3 day experiment (figure 4-47, 4-48, 4-49).

There was only one comparison resulting in a significant difference and that occurred at the 2 hour 75% duty cycle for area, in which the 3 day area was significantly higher than the 1 day area.

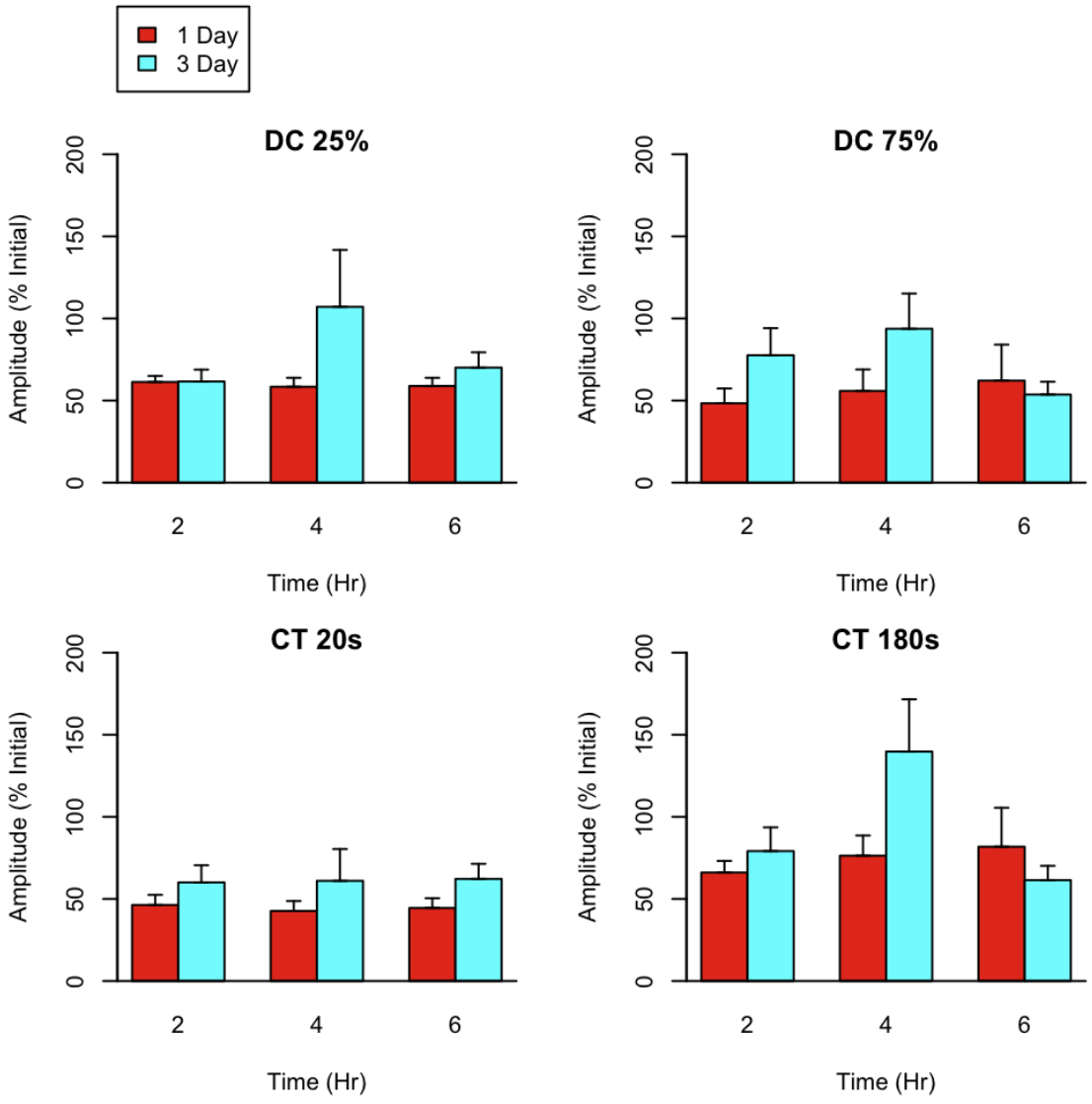


Figure 4-47: Average M-wave amplitude comparison of the specified cycles throughout the 1 and 3 day experiment for each duty cycle and cycle time group.

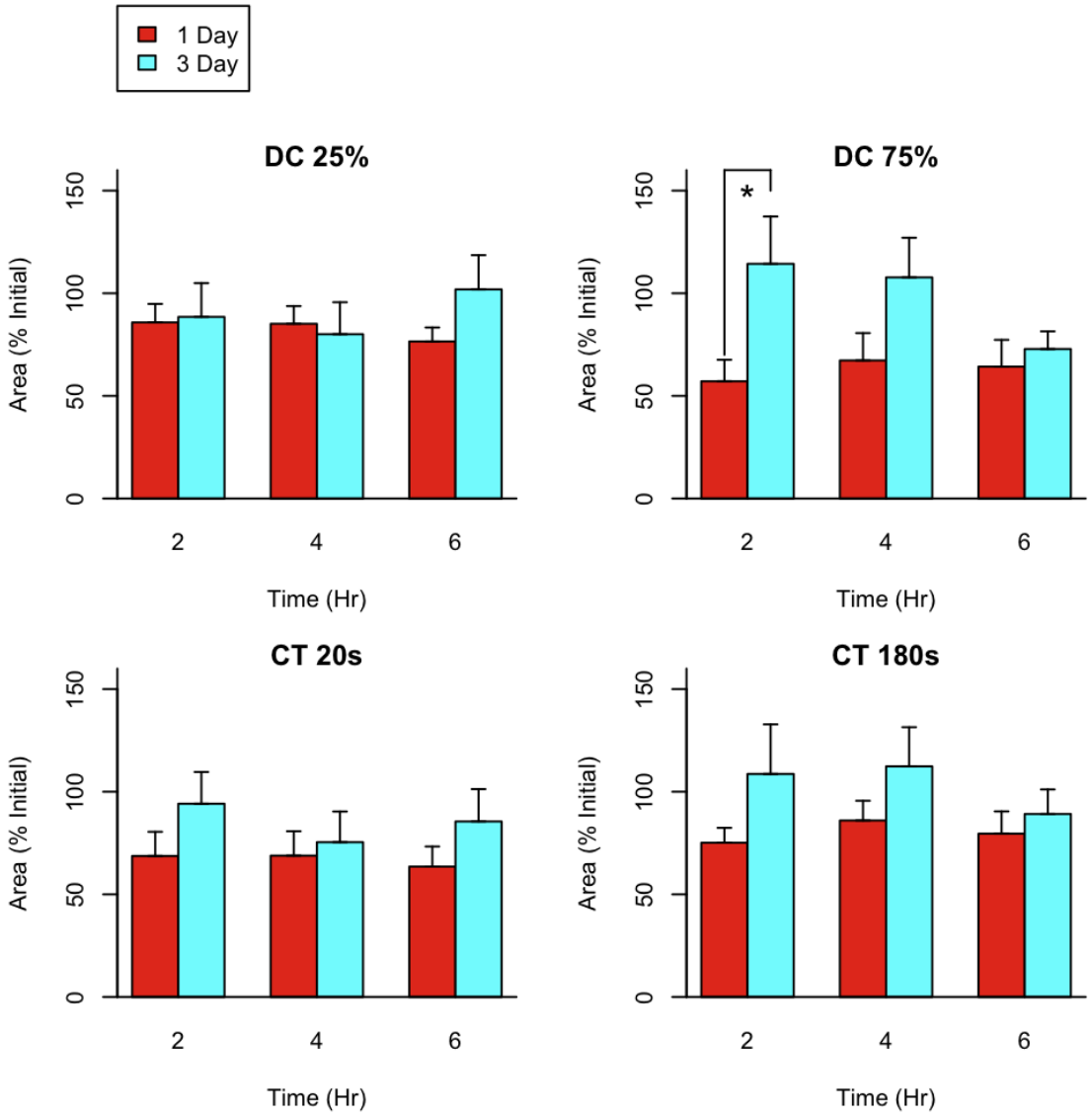


Figure 4-48: Average M-wave area comparison of the specified cycles throughout the 1 and 3 day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

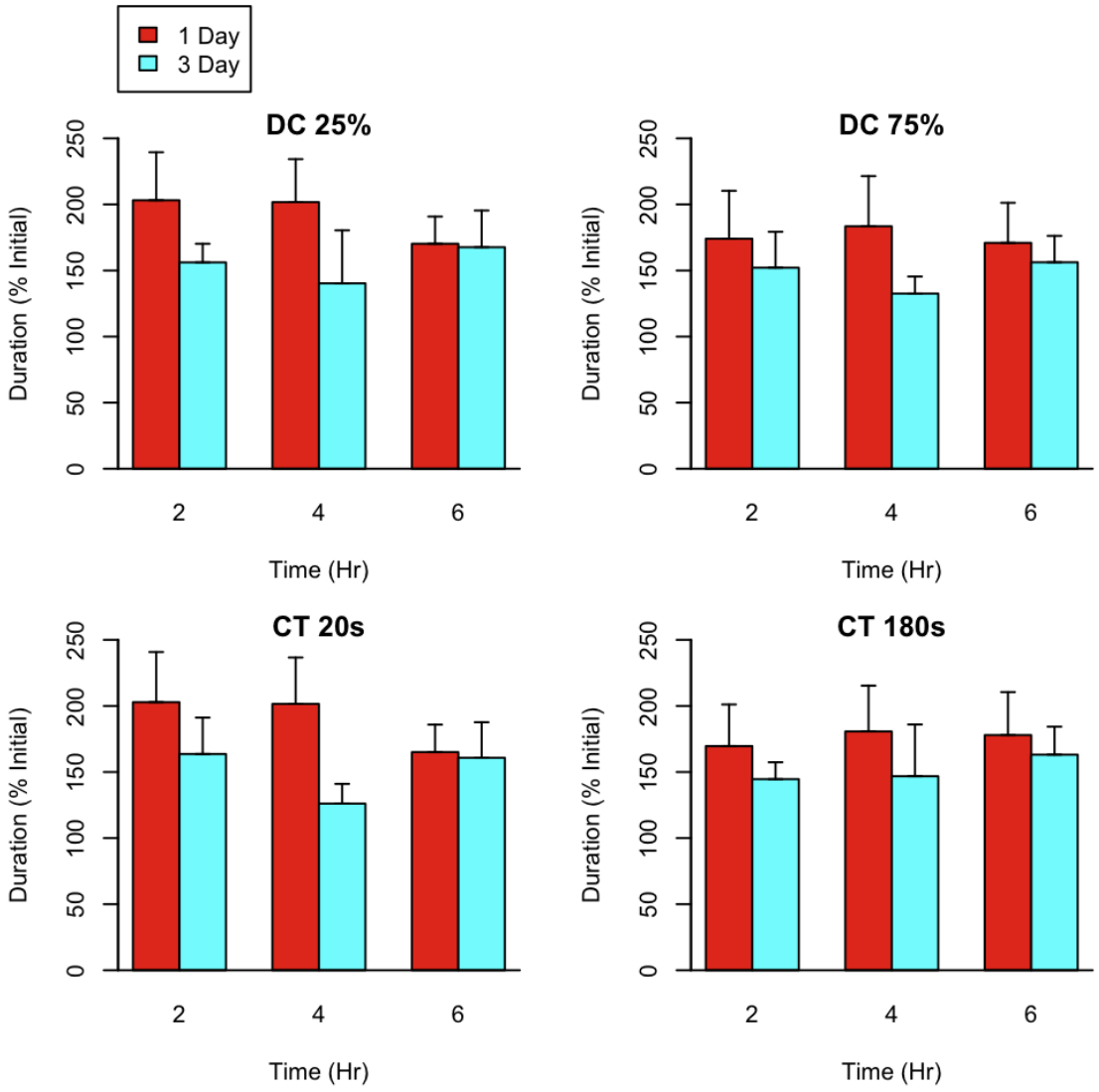


Figure 4-49: Average M-wave duration comparison of the specified cycles throughout the 1 and 3 day experiment for each duty cycle and cycle time group.

4.7.3.3 Work/Rest Group Comparison From the Beginning to the End of Each Cycle

Overall, the M-wave amplitude decrease within each cycle was greater in the 1-day experiment compared to the 3-day experiment (figure 4-50). This was significant at 2 hours. M-wave area also showed a significant difference at 2 hours with the 1-day experiment having a larger percent decrease from the beginning to the end of the cycle. At 6 hours, the 1-day experiment decrease in

area was less than the 3-day experiment but this result was not significant. There were no significant differences between the 1-day experiment and 3 day experiment for duration of the M-wave.

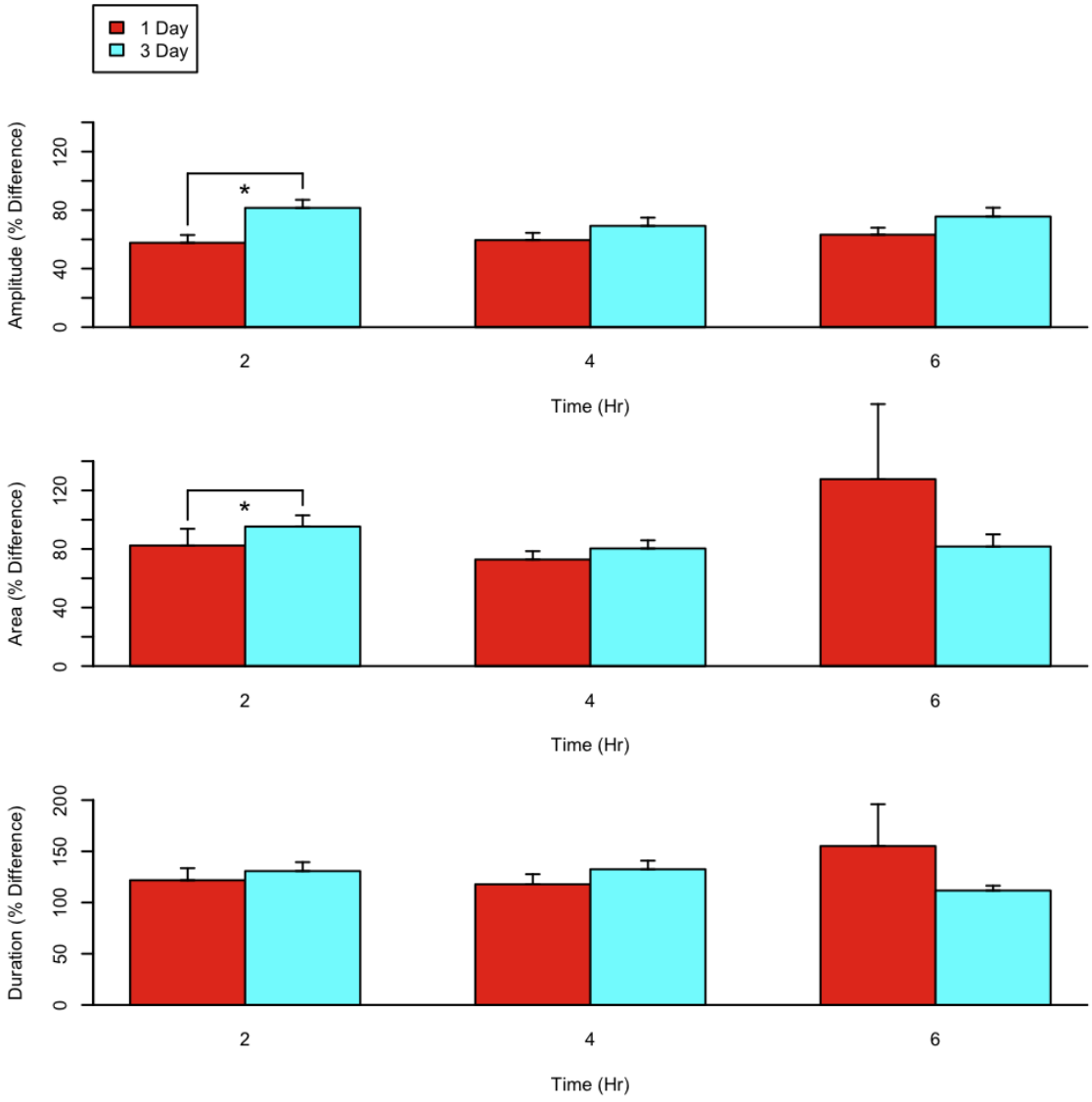


Figure 4-50: Overall average M-wave amplitude, area, and duration comparing the beginning to end of each of the specified cycles throughout the 1 and 3 day experiments. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

Generally, amplitude change from the beginning to end of a cycle was greater for the 1 day experiment compared to the 3 day experiment (figure 4-51).

The differences from the beginning to the end of each cycle for group A and B at 6 hours were significant comparing the 1 day and 3 day experiment. Also, group B had significant differences at 4 and 6 hours between the 1 and 2 day experiments.

M-wave area changes within a cycle were greater for the 1 day experiments at each time point for groups A and B (figure 4-52). At 6 hours for group B this change was significantly different. Group C had similar area decreases between the 1 and 3 day experiments for 2 and 4 hours. However, at 6 hours, there was a larger but not significant percent increase in area for the 1-day experiment. Group D had larger, but not significant, percent decreases in area for the 3-day experiment compared to the 1-day experiments.

M-wave duration increases were greater for the 3-day experiment at each cycle in group A compared to the 1-day experiment (figure 4-53). In groups B and C, percent duration change was greater for the 1-day experiment at 2 and 4 hours but not at 6 hours. None of these differences were significant.

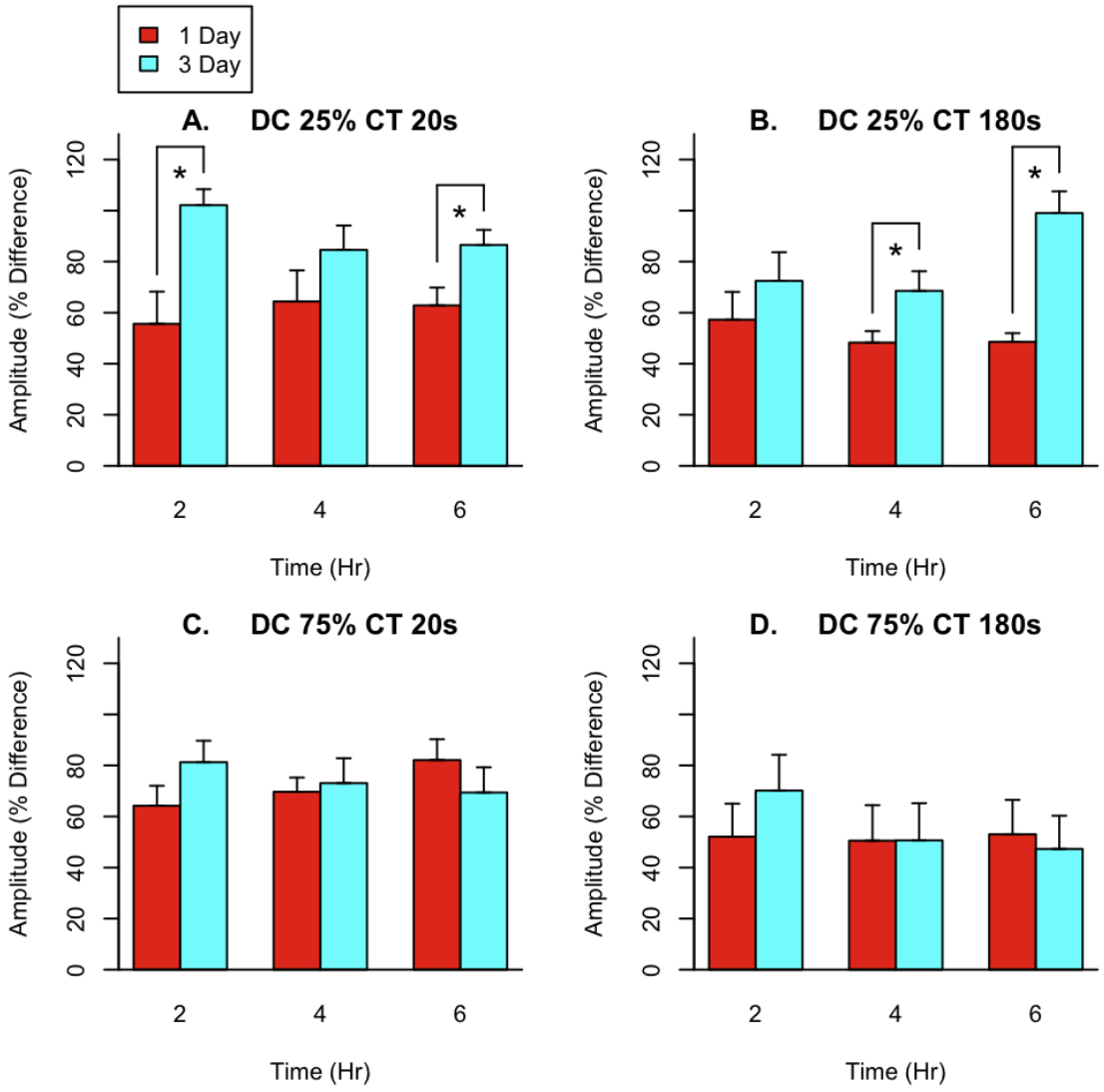


Figure 4-51: Average M-wave amplitude comparison of the beginning to end of each of the specified cycles throughout the 1 and 3 day experiments for each work/rest group. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

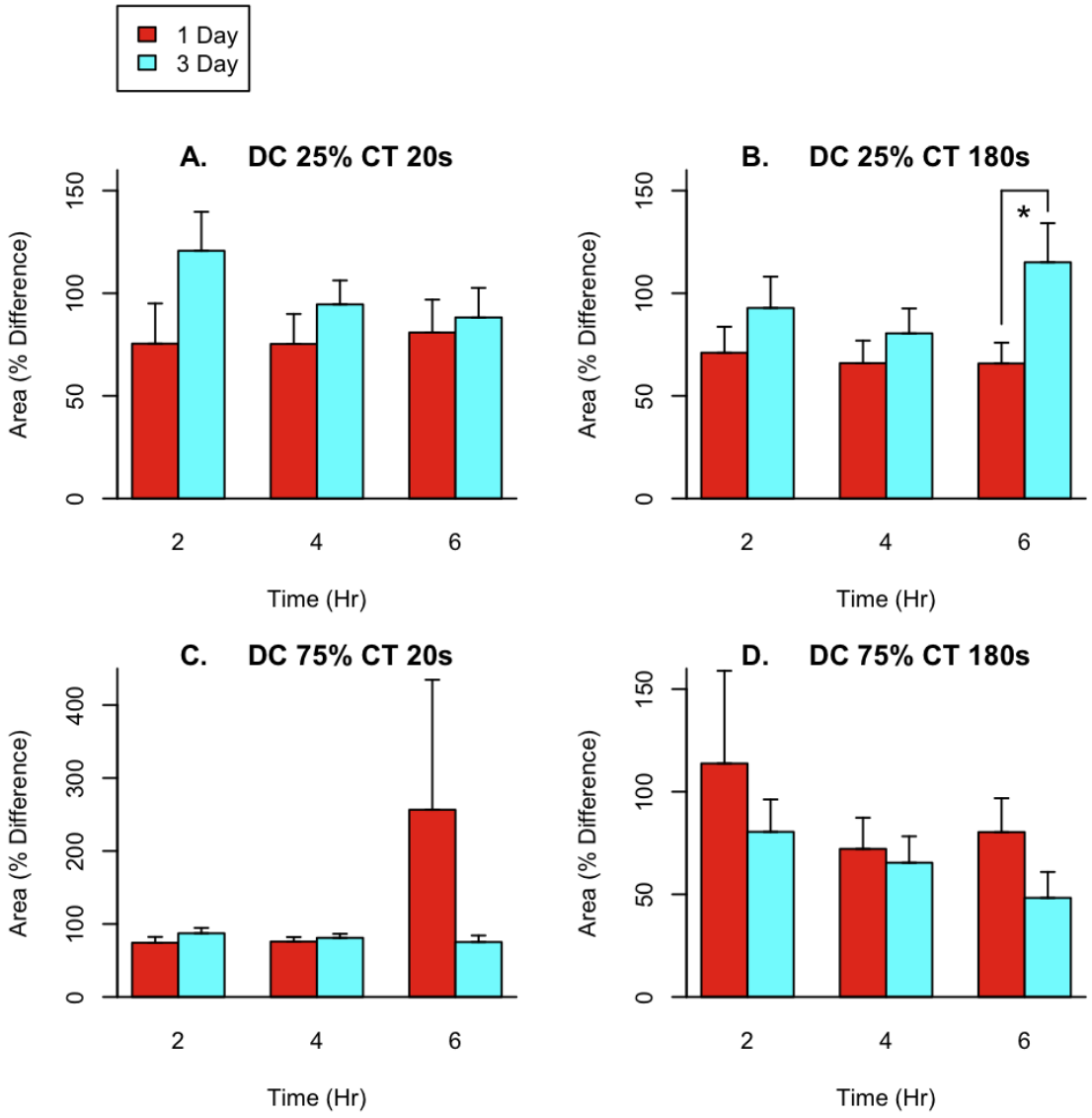


Figure 4-52: Average M-wave area comparison of the beginning to end of each of the specified cycles throughout the 1 and 3 day experiments for each work/rest group. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

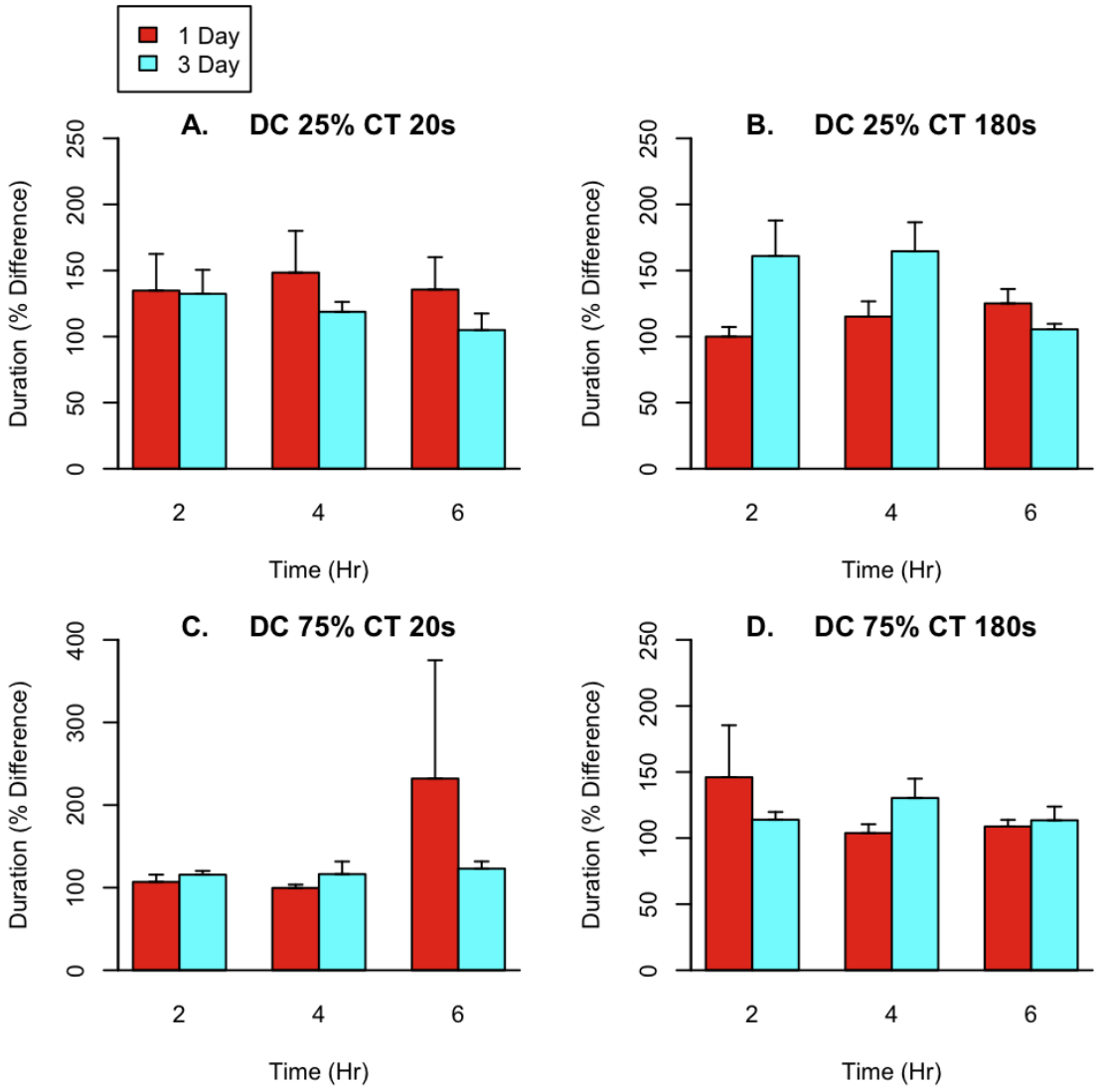


Figure 4-53: Average M-wave duration comparison of the beginning to end of each of the specified cycles throughout the 1 and 3 day experiments for each work/rest group.

4.7.3.4 Duty Cycle and Cycle Time Comparison From the Beginning to the End of Each Cycle

The 1-day experiment had larger changes in M-wave amplitude than the 3-day experiment at each time point for the 25% duty cycle and the 20s and 180s cycle time groups (figure 4-54). These differences were significant at 2 and 6 hours for the 25% duty cycle and at 2 hours for the 20s cycle time group. The 75% duty cycle group resulted in the amplitude changes for the 1-day experiment

to be larger at 2 hours, smaller at 6 hours and the same at 4 hours. None of these differences were significant.

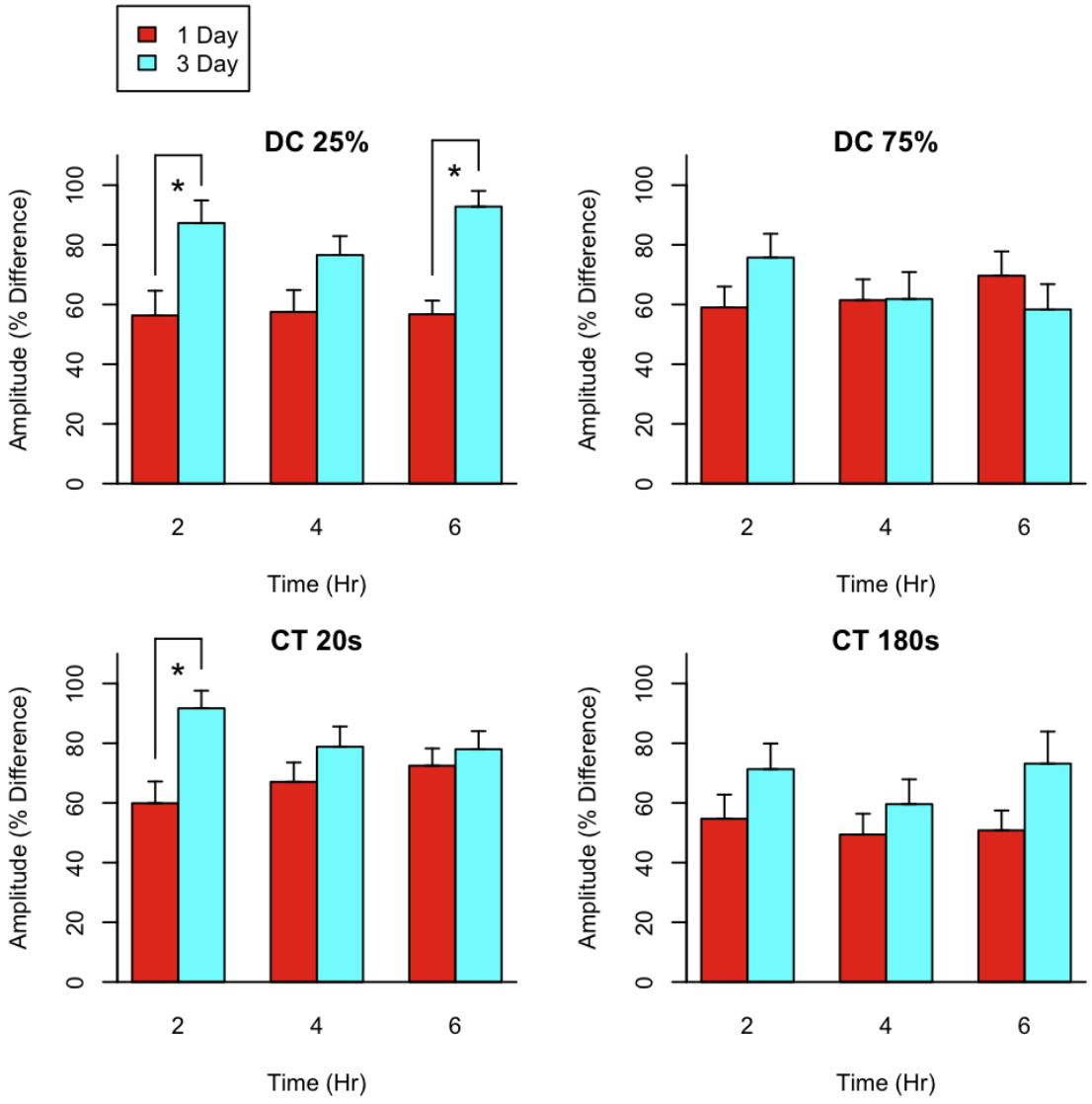


Figure 4-54: Average M-wave amplitude comparison of the beginning to end of each of the specified cycles throughout the 1 and 3 day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

Similar to amplitude, the 1-day experiment resulted in a larger percent change in area compared to the 3-day experiment for each time point on the 25% duty cycle (figure 4-55). Only the 2-hour cycle was significantly different. The

remaining duty cycle and cycle time groups did not have significant differences between the 3 day and 1 day experiments for area.

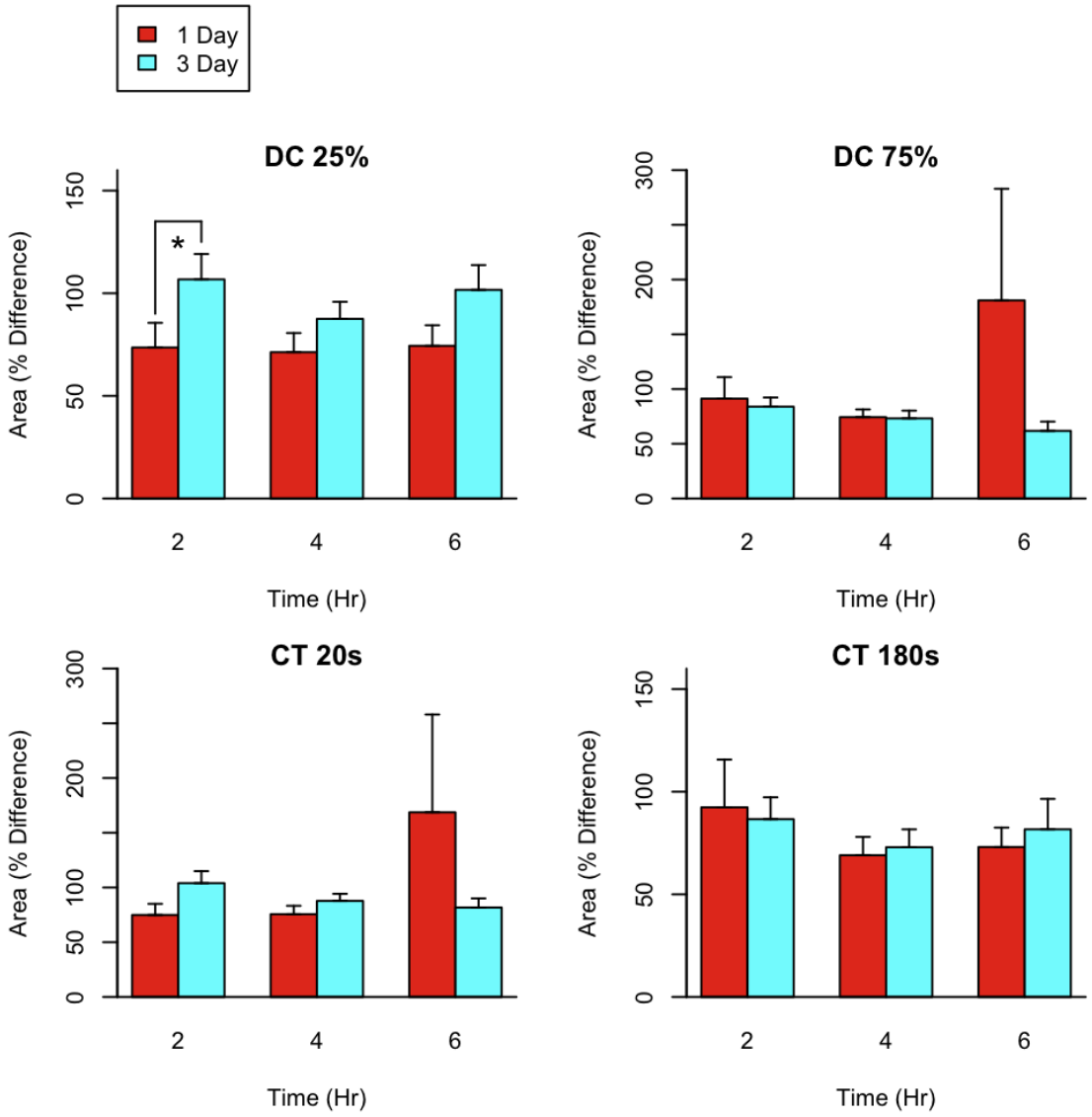


Figure 4-55: Average M-wave area comparison of the beginning to end of each of the specified cycles throughout the 1 and 3 day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

There was no clear trend for changes in duration from the beginning to the end of each cycle, when comparing the 1 day and 3 day experiments (figure 4-56). Within each duty cycle and cycle time groups, the greater increase in

duration varied at different time points. Only the 180s cycle time at 4 hours resulted in a significant difference, with the 1-day experiment having a larger change in duration.

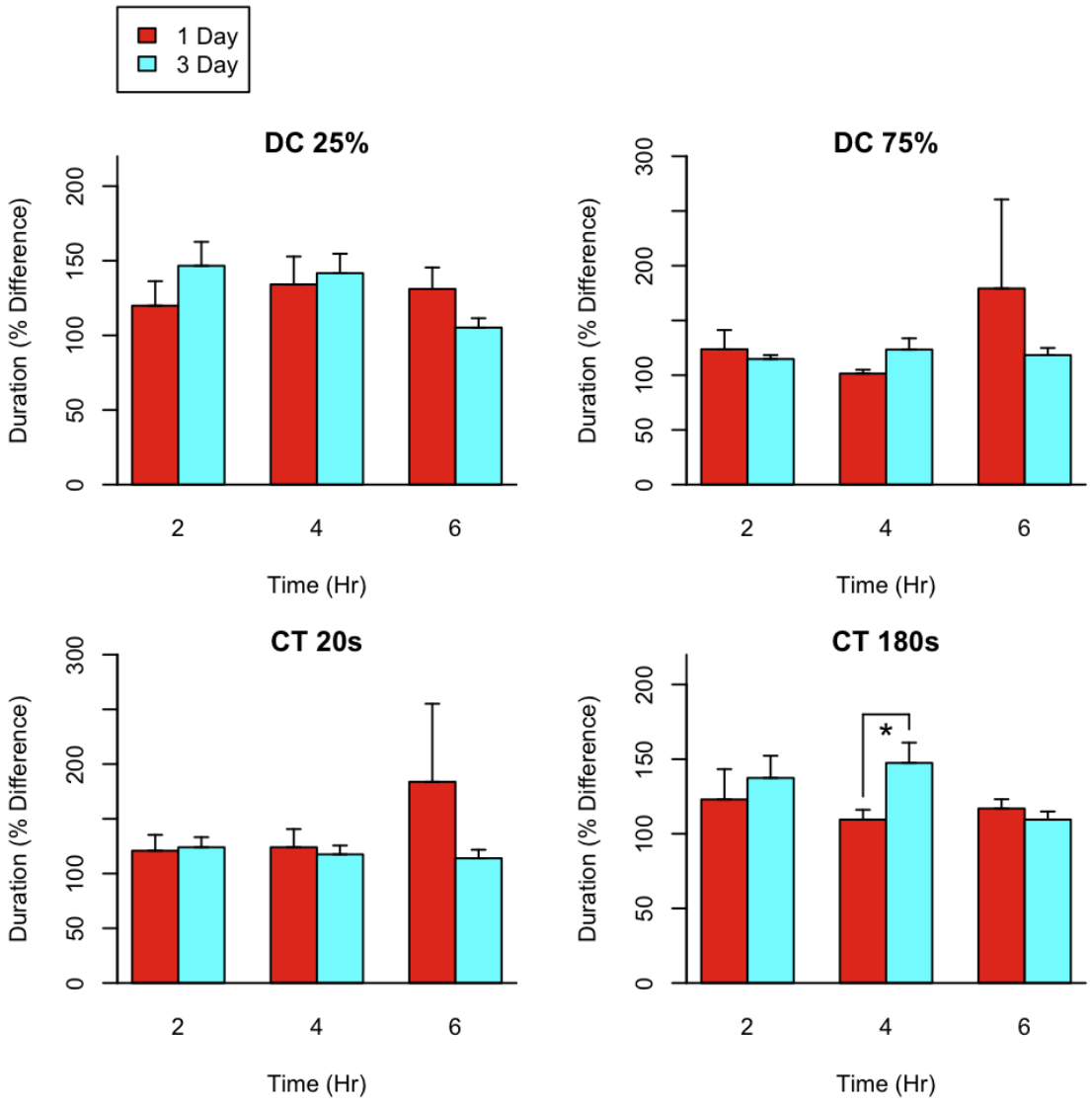


Figure 4-56: Average M-wave duration comparison of the beginning to end of each of the specified cycles throughout the 1 and 3 day experiment for each duty cycle and cycle time group. Significant differences ($p<0.05$) between days are indicated by brackets and the * symbol.

4.7.4 Discussion

Since the 3-day experimental results were calculated as a percent of the initial cycle for each day, in order to make a comparison with the 1-day experiment, the data was calculated as a percentage of the initial cycle. In general, the amplitude and area for the 1 day experiment at each time point had a lower percentage of the initial value, while duration had a higher percentage of the initial value compared to the 3 day experiment. This was expected since the M-wave signal for the 3-day experiment was diminished based on the discussion in section 4.5. Thus, there was a greater potential for change in the M-wave signal for the 1-day experiment group.

There were only 2 comparisons in all of the time points in all of the groups that resulted in significant differences. This indicates that the percent change between the 1-day and 3-day experiments were similar. The duty cycle and cycle time comparisons between the 1 day and 3 day experiments also had similar percent of the initial values with only 1 time point resulting in a significant difference.

These results do not provide substantial support for employing this method of stimulation and recording over several days. Although long-term electrode experiments are performed, they do not consist of both indwelling stimulating and recording electrodes, leaving questions as to the strength and stability of the signal over time. This study has demonstrated that the methods employed reduce the signal strength of the M-wave most likely due to tissue healing around the electrodes, making it difficult to analyze the data. Thus, the larger change in the 1 day M-wave data is most likely due to the stronger signal

in that study rather than due to increased fatigue. Comparing the M-wave values from the beginning to the end of the experiment for each day or from the beginning to the end of a cycle is the most feasible method of data assessment.

In summary, measuring M-waves with indwelling stimulating and recording electrodes is reliable for a 1-day study. In the 1-day experiment group C (DC 75% CT 20s) resulted in the greatest fatigue, suggesting that high duty cycle with short rest allowance is not recommended. Further investigation is required to determine the reason for the diminished signal between the surgery day and the start of the 3-day experiment. However, comparing the 1 day and 3 day M-wave data did not provide significant differences.

CHAPTER 5 – HISTOLOGICAL ASSESSMENT OF THE MEDIAL LONGISSIMUS RAT MUSCLE DUE TO *IN VIVO* ELECTRICAL STIMULATION

5.1 Introduction and Rationale

Electrical stimulation experiments are a common method for inducing muscle injury. Typically, an eccentric contraction is employed during the stimulation to ensure that injury will occur (Lieber et al., 1994). However, there is evidence that muscle injury can occur due to electrical stimulation without employing eccentric contractions (Lexell et al., 1993).

Also, although it has been found that continuous stimulation is more injurious to the muscle than intermittent stimulation, intermittent stimulation can produce injury (Clausen et al., 2004). Clausen et al., (2004) stimulated extensor digitorum longus and soleus muscles for 1 s with a 3 s rest and repeated for up to 2 minutes. In the current study, various work/rest ratios were employed for as long as 6 hours.

It has been found that in animal models fast twitch muscle fibers are injured more easily than slow twitch muscle fibers (Clausen et al., 1998). Electrical stimulation with or without eccentric contraction utilizes fast twitch muscles, due to their higher force capabilities. These fibers fatigue quicker than slow twitch muscle fibers and this fatigue may be the reason for injury. In contrast, low force prolonged contractions, as found in cleaning employees, resulted in damage to slow twitch muscle fibers (Larsson, 2000). Therefore, utilizing a variety of stimulation protocols for a prolonged duration may affect different fiber types.

The assessment of muscle injury can involve applying various staining techniques and observing the muscle sections under a microscope as reviewed in chapter 3. Briefly, the immediate effect of muscle damage is an inflammatory response. H&E stains are commonly used to assess inflammation by counting the number of white blood cells under a light microscope. A more specific look at the inflammatory process would analyze the ED1 macrophage with a specific immunohistochemistry stain in order to determine the stage of the inflammatory process. NADH can be utilized to determine the proportion of fast compare to slow twitch muscle fibers, as well as injury to slow twitch fibers. Structural damage to the cell can be evaluated with a desmin stain and then regeneration of the fiber can be observed with the presence of vimentin.

5.1.1 Specific Aim VI

Previous studies (chapter 4) have shown that electrically stimulating the medial longissimus muscle at various duty cycles and cycle times result in fatigue, as measured by M-waves. An additional outcome of electrical muscle stimulation may be muscle injury. Thus, the purpose of this study was to determine the effect of various stimulation protocols on muscle injury.

5.2 Methods

5.2.1 Tissue Preparation

The rats were euthanized 4 days after the start of the stimulation protocol with an overdose of sodium pentobarbital (120mg/kg, IP). Upon sacrifice, the medial longissimus muscle was removed and trimmed of fascia. The caudal portion of the muscle was mounted transversely on a cork disc and held in place by optimal cutting temperature (OCT) compound. The cork with the specimen

was frozen in isopentane cooled in liquid nitrogen. Immediately after harvesting the tissue, the samples were sectioned transversely (10μm thick) in a cryostat (Leica CM3050, Leica Microsystems, Nussloch, GmbH). Between 2 and 5 sections were collected on Superfrost®/Plus glass slides (Fisher Scientific) for all analyses. The slides were stored at -80°C until they were ready to be stained.

5.2.2 Histology

A routine hematoxylin and eosin (H&E) procedure was applied to stain for general morphological damage such as swollen and necrotic fibers and inflammation with evidence of invasion of mononuclear cells. The muscle sections were processed as follows:

1. Stained in Hematoxylin Gill 3 for approximately 2 minutes.
2. Washed 3 times in tap water
3. Placed in differentiating solution for 10 seconds
4. Washed 3 times in tap water
5. Placed in Scott's tap water for 10 seconds
6. Washed 3 times in tap water
7. Counterstained in Eosin solution for 45 seconds
8. Washed 3 times in tap water
9. Dehydrated through graded alcohol, cleared in xylene, and coverslipped using Permount (Fisher Scientific).

5.2.3 Histochemistry

A determination of mitochondrial damage, known as moth-eaten fibers can be made with a nicotine-adenine-dinucleotide-tetrazolium reductase (NADH-TR) stain (Dubowitz and Sewry, 2007). Fibers give the appearance of being moth-eaten due to disruption of myofibrils, indicating damage to mitochondria. Fiber types were also determined with NADH-TR. Type I or slow oxidative fibers stain darker than the fast twitch Type II fibers. The muscle sections were processed as follows:

1 Placed 1 – 2 drops of incubating solution on the section.

2 Incubated for 30 minutes at 37°C.

3 Rinsed in distilled water.

4 Fixed in 15-20% formalin solution for 10 minutes.

5 Rinsed in distilled water.

6 Coverslipped using Permount (Fisher Scientific).

5.2.4 Immunohistochemistry

There were 3 immunohistochemistry techniques employed to the sections: ED1, Desmin, and Vimentin. The ED1 stain identified the ED1 macrophages occurring in the inflammatory process. Desmin is a protein responsible for the structure of the cell and a lack of desmin staining indicated injury to the muscle fiber. Regeneration of the muscle fiber was demonstrated when there was evidence of the protein Vimentin. The muscle sections were processed as follows:

1 Incubated in 0.3% hydrogen peroxide to block endogenous peroxide activity.

2 Rinsed in phosphate buffered saline (PBS) three times.

3 Incubated overnight at -0.4°C in monoclonal antibodies to either desmin (D33, 1:100, Dako, code No. M0760, Carpenteria, CA), vimentin (V9, 1:10, DAKO code No. M0725, Carpenteria, CA), or ED1 macrophages (CD68, 1:250, Millipore catalogue No. MAB1435, Temecula, CA).

4 Rinsed in phosphate buffered saline (PBS) three times.

5 Incubated for 30 minutes at room temperature in Biotinylated anti-mouse IgG.

6 Rinsed in phosphate buffered saline (PBS) three times.

7 Incubated for 30 minutes at room temperature in ABC solution.

8 Rinsed in phosphate buffered saline (PBS) three times.

9 Incubated for 2 minutes at room temperature in 3, 3'-diaminobenzidine (DAB) and hydrogen peroxide.

10 The ED1 sections were then counterstained with hematoxylin.

11 Dehydrated through graded alcohol, cleared in xylene, and coverslipped using Permount (Fisher Scientific).

5.2.5 Data Analysis

Each histological, histochemical, and immunohistochemical analyses consisted of three slides from three regions of the muscle. This was accomplished by alternating stains for each successive slide. Once the slides were completed for each stain, the process was repeated two more times from more rostral locations of the muscle. Table 5-1 provides the order in which the slides were obtained.

From the 2 – 5 sections on each slide, one was used for analysis, with the remaining sections as back up. Tissue sections were evaluated under a light microscope (Leica DMLB, Leica Microsystems, Wetzlar, GmbH). The area to be analyzed was determined by viewing the sections under 50x magnification. A higher magnification was then utilized to view the details of the section within these boundaries. Digital pictures of these areas were obtained using a digital camera system (ProgRes C7, Jenoptik, GmbH) attached to the microscope.

Table 5-1: Slide staining order

	Stain				
	H&E	**NADH**	**ED1**	**Desmin**	**Vimentin**
Slide #	1	2	3	4	5
	6	7	8	9	10
	11	12	13	14	15

For H&E slides the sections within this 50x magnification boundary were observed at a 200x magnification under the microscope. The area of the section observed under 200x magnification was termed a high power field (HPF). There were an average of approximately 6 HPF analyzed in each section. Within each HPF a search was conducted for signs of inflammation, with the evidence being the appearance of white blood cells. Confirmation of a white blood cell was determined by observing the cell under 400x magnification. A picture was taken of the positively identified white blood cell and the magnification was returned to 200x. The slide was then moved to the next HPF location and the search for white blood cells continued. There were an average of 6 HPF per slide. The

number of white blood cells were counted and recorded as a percentage of HPF per the following equation.

Equation 1: Percentage of white blood cells = (Number of white blood cells/Number of high power fields) * 100

Images of the NADH stains were obtained at 100x magnification and the number of lightly stained fibers and dark stained fibers were counted and recorded as a percentage of fast twitch and slow twitch fibers per section. Also, indication of a fiber that showed signs of damage was magnified 400x and an image was captured if it was determined that it was a moth eaten fiber. These fibers were counted and recorded as a total of moth eaten fibers for the 3 sections analyzed.

The immunohistochemistry stains: ED1, desmin, and vimentin were all observed under 100x magnification. Under this magnification if there was any indication of a positive result the image under the microscope was magnified to 400x. Once a positive result was confirmed, a picture was taken and the microscope was returned to 100x magnification and the search continued. The number of ED1 macrophages were counted and reported as a total of the 3 sections analyzed. Also, the number of fibers showing a lack of desmin staining and positive vimentin staining were counted and reported as a total of the 3 sections analyzed, respectively.

5.2.6 Positive Control

Bupivacaine, a local anesthetic, has been frequently used to induce experimental muscle injury, which follows a reproducible, chronological sequence of muscle injury and regeneration (Nonaka, et al., 1983; Kaminska and

Fidzianska, 1996; Wakata, et al., 2001; Horiguchi, et al., 2002; Zink and Graf, 2004; and Politi, et al., 2006). Thus, the results of the injection of bupivacaine were used as a standard to compare to the histological, histochemical, and immunohistochemical analyses due to electrical stimulation. This was necessary in order to ensure the accuracy of the current stimulation-induced injury analyses.

This procedure utilized 1 rat. Under anesthesia, the low back of the rat was shaved and prepped with Betadine. A small incision no more than 2 cm long, using a #10 blade, was made in the skin overlying the lumbar portion of the spinal column. This part of the procedure was no different than the surgical protocol used for the stimulation experiments.

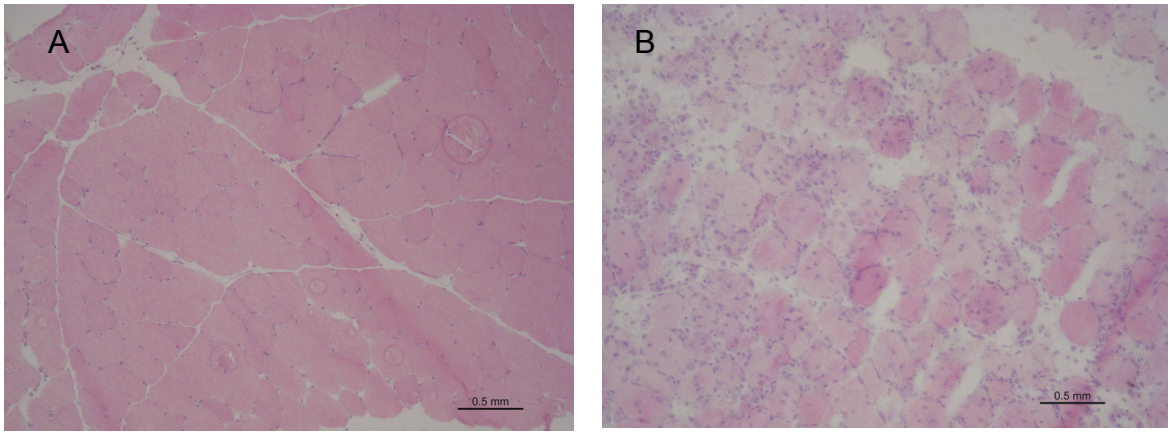
A thin-gauge needle was inserted into the left medial longissimus muscle, injecting 0.5% bupivacaine in 1.5ml 0.9% sterile saline. In the right medial longissimus muscle 1.5ml 0.9% sterile saline was injected to serve as control. The skin was sutured and butorphanol (2mg/kg, sc) was administered for pain relief. The rat was sacrificed on the 4th day after bupivacaine injection and the tissues were harvested for histological, histochemical, and immunohistochemical evaluation.

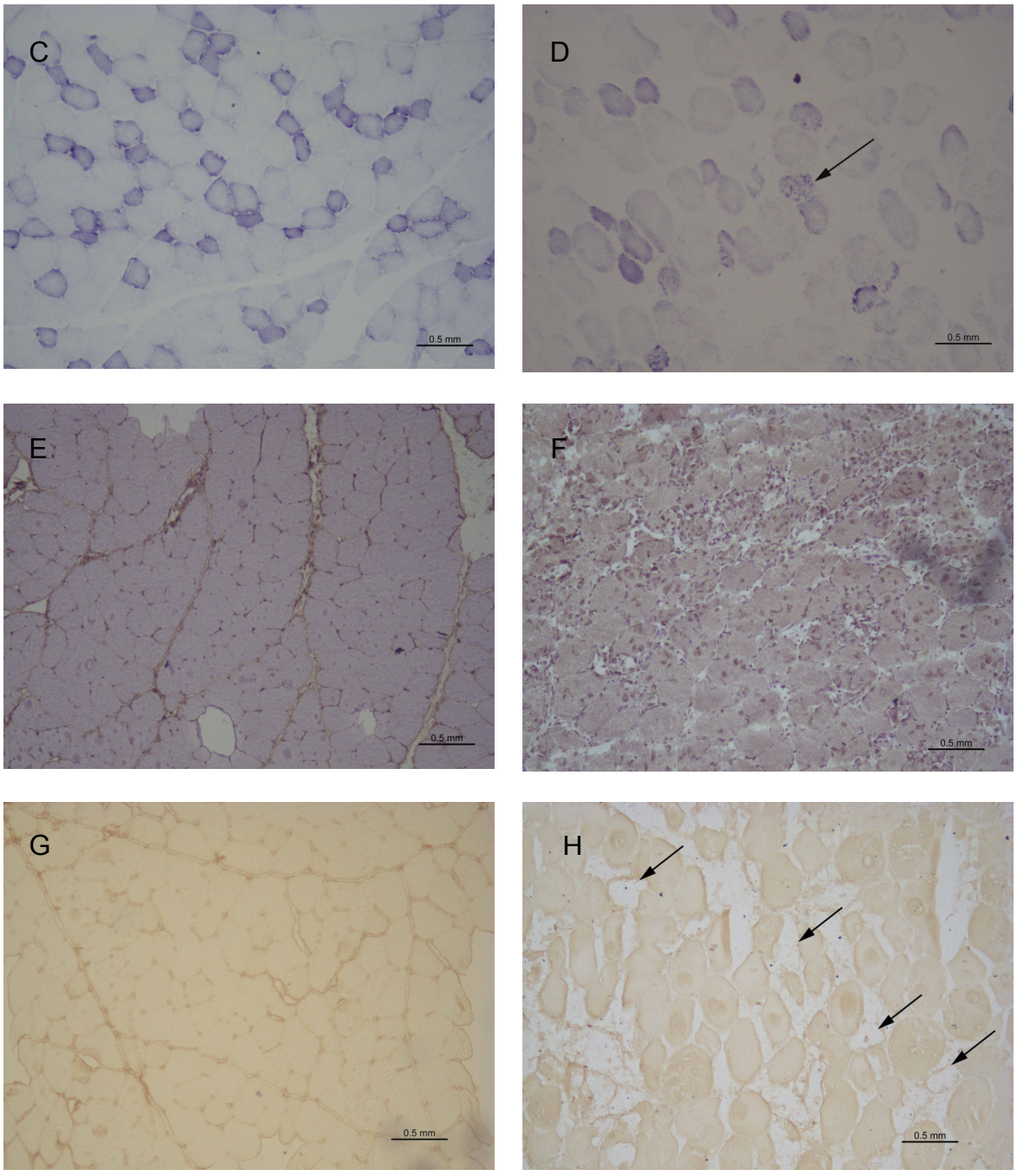
Figure 5-1 displays the resultant stains for the bupivacaine injured muscle and the saline control. The H&E saline injected muscle image (figure 5-1 A) is an indication of a healthy, non-injured muscle. In comparison, figure 5-1 B is an H&E image of a muscle in the same rat injected with bupivacaine. There is

evidence of a substantial inflammatory response. Several fibers are engulfed in white blood cells. There is also evidence of necrotic fibers.

The NADH-stained sections (figure 5-1 C&D) show contrast between a saline-injected non-injured muscle and a bupivacaine-injured muscle. The arrow in figure 5-1 D indicates a moth-eaten fiber, whereas, there are no fibers in figure 5-1 C with similar damage.

Many of the inflammatory cells found in figure 5-1 B may be ED1 macrophages as evidence in the ED1 stained figure 5-1 F. The corresponding stain in figure 5-1 E has no indication of macrophages. The desmin stained slides also show a difference between the saline-injected (figure 5-1 G) and bupivacaine-injected (figure 5-1 H) muscles. In G, the muscle fibers are intact, while the arrows in H indicate areas of the muscle where fibers are absent. Finally, the vimentin stain in figure 5-1 I reveals outlines of the individual muscle fibers with a light stain of the interior of the fibers. The bupivacaine-injured muscle (figure 5-1 J) shows signs of regeneration due to the dark stained fibers.





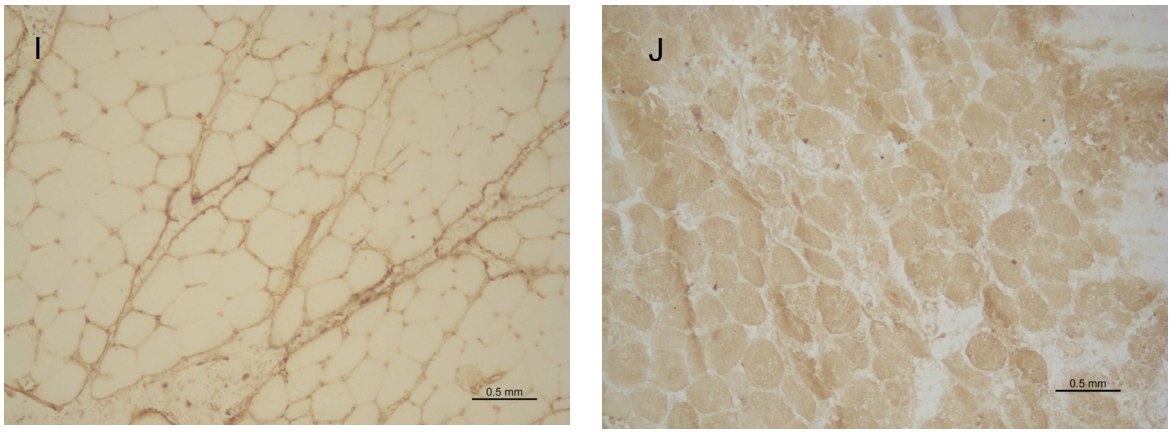


Figure 5-1: Stains of positive control rat muscle. The left column (A,C,E,G,I) represents the saline injected muscle, while the right column (B,D,F,H,J) represents the bupivacaine-injected muscle. **A-B**) H&E, **C-D**) NADH (arrows indicate moth-eaten fibers, **E-F**) ED1, **G-H**) Desmin (arrows indicate lack of desmin, I-J) Vimentin. All images taken at 100x magnification.

5.2.7 Statistical Analysis

The analyses were performed on a total of 60 Male Sprague-Dawley rats (400-450g). The mean and standard error was calculated for each of the histological, histochemical, and immunohistochemical analyses for each experimental and sham group. The analyses included the percentage of white blood cells per high power field (H&E), number of moth eaten fibers (NADH), number of ED1 macrophages, number of fibers lacking desmin, and number of fibers indicating the occurrence of vimentin.

A comparison between each work/rest group using the Mann-Whitney Test was then performed in the 1-Day experiment and the 3-Day experiment, separately. The groups were separated into duty cycle and cycle time groups and the Mann-Whitney tests were repeated on these groups. Then a Mann-Whitney test was performed comparing the 1 Day and 3 Day work/rest groups. For example, the comparisons were performed between the groups containing

the same stimulation protocol but differing between days. This was also performed between the duty cycle and cycle time groups.

An alpha level of $p<0.05$ was considered significant for these analyses. All statistical procedures were performed in PASW Statistics v18.1 (SPSS, Inc Chicago, IL). The nonparametric tests were used due to non-normal distribution.

5.3 Results

5.3.1 Image Analysis

Figure 5-2 provides sample images of each of the stains employed in the experiment. The H&E stain in figure 5-2 A shows a healthy muscle with no evidence of inflammatory cells or damaged fibers. This type of image was typically found in all of the rat slides. There was not conclusive evidence that any of the fibers were swollen or atrophied and thus statistical analysis was not performed on this category of injuries. In figure 5-2 B the arrows indicate there is evidence of inflammatory cells. Although these cells did not occur as frequently as the positive control in section 5.2.6, there were enough examples found to warrant statistical analysis.

The images in 5-2 C, D, E, and F were typical images found in all of the rat slides for the specified stains. The NADH assessments (figure 5-2 C) did not find evidence of moth eaten fibers, but provided excellent differentiation to count the number of fast-twitch and slow twitch fibers. There were no positive confirmations of ED1 macrophages (figure 5-2 D). The desmin stains (figure 5-2 E) provided images of complete fibers, with no white spaces where fibers should have been. Finally, the vimentin stains (figure 5-2 F) did not provide any evidence of this protein.

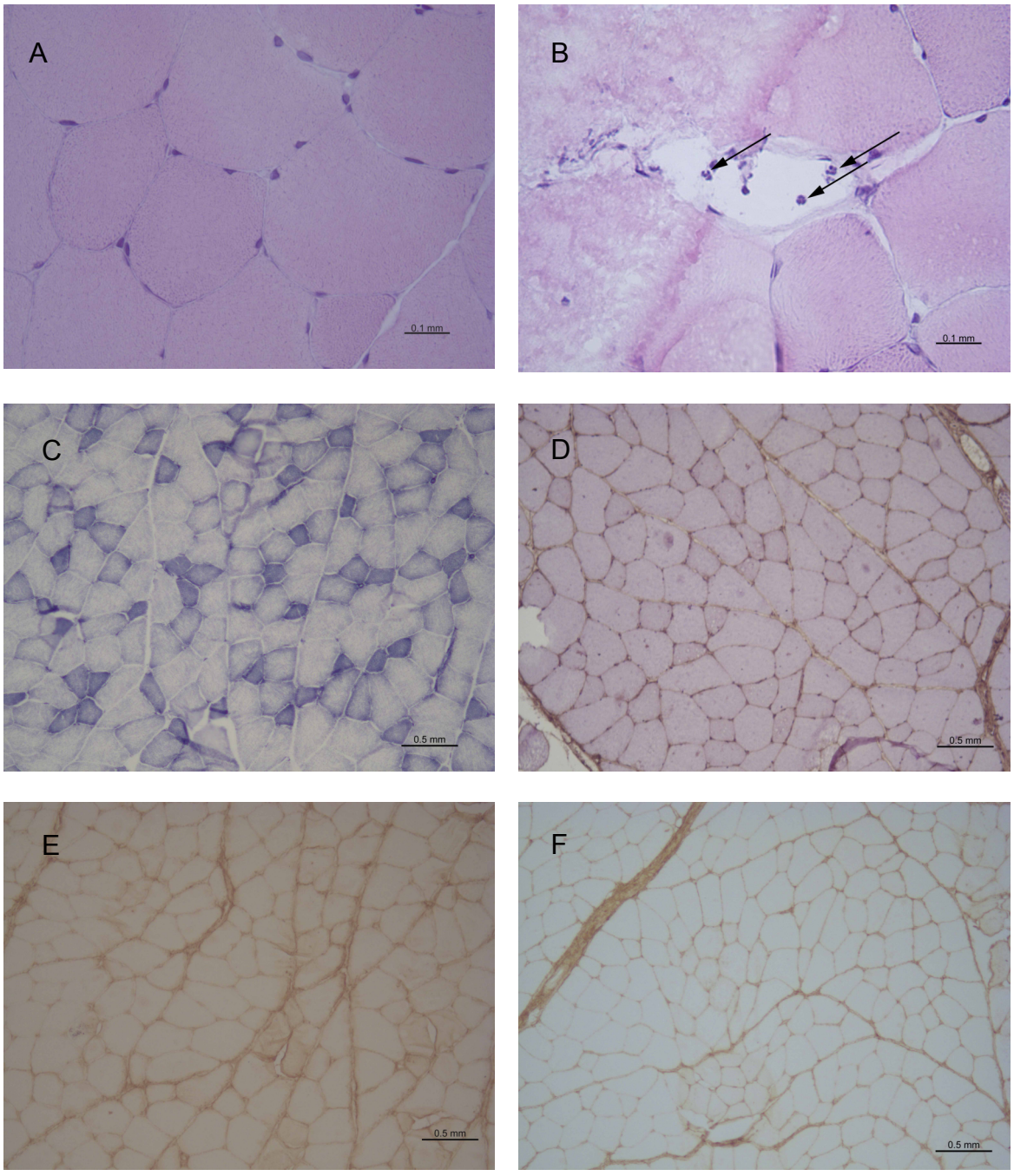


Figure 5-2: Stains of selected tissue of experimental rats. **A-B**) H&E stain with arrows indicating inflammatory cells. **C**) NADH, **D**) ED1, **E**) Desmin, **F**) Vimentin. H&E images (A,B) taken at 40x magnification. NADH, ED1, Desmin, and Vimentin images (**C,D,E,F**) taken at 100x magnification.

A benefit of NADH is that it stains slow twitch type I fibers dark and fast twitch type II fiber light. This stain was employed on all of the stimulated

muscles. Within the 10x high power field a count was performed to determine the number of type I and II fibers contained within the medial longissimus muscle of the rat. The rats were pooled and it was found that the fast twitch type II fibers outnumber the slow twitch type I fibers by approximately a 2:1 ratio (figure 5-3).

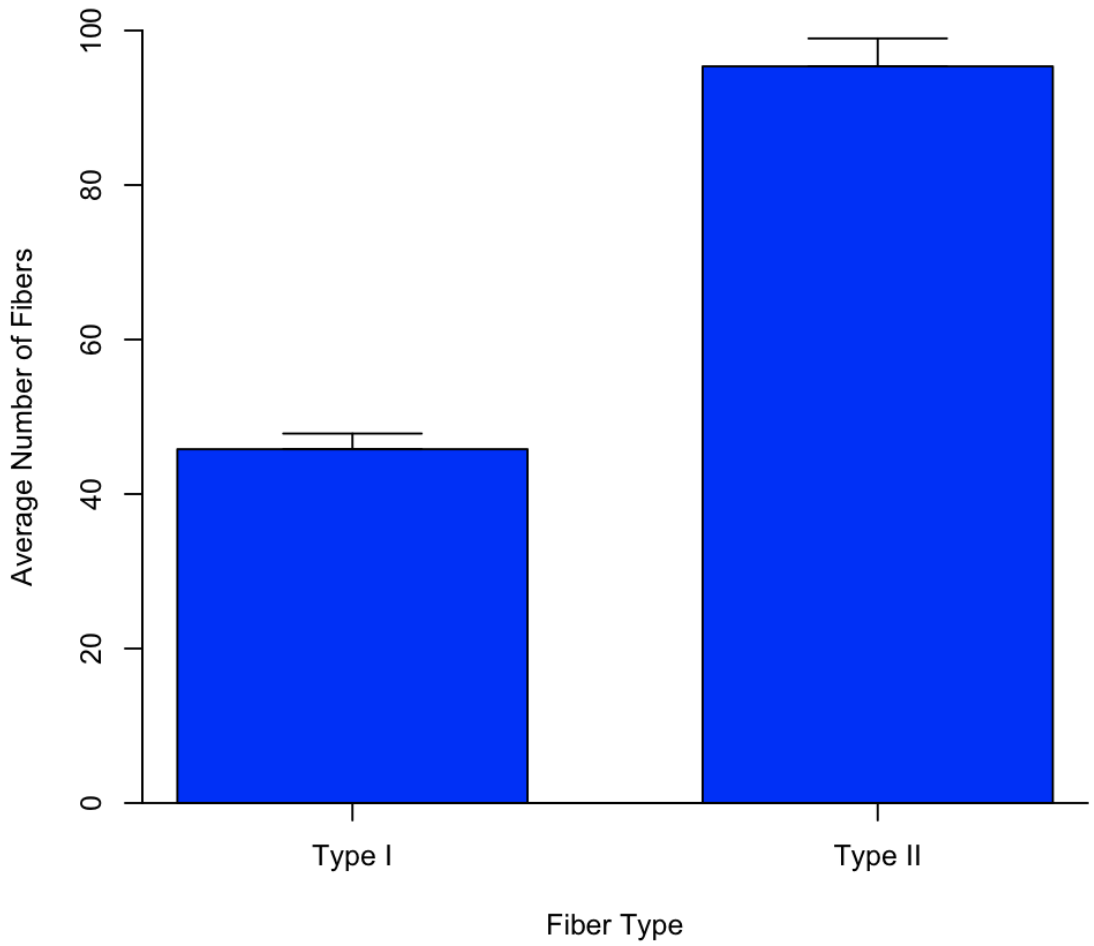


Figure 5-3: Comparison of fiber types I and II for all rats utilizing the NADH stain

5.3.2 H&E Analysis of the 1 Day Experiment

The sham group of rats had the lowest percentage of white blood cells compared to all 4 of the work/rest groups (figure 5-4). The DC 25% CT 180s

group resulted in the highest percent of white blood cell/high power field and was the only work/rest group that was significantly different than the sham group. There was also a significant difference between the DC 25% CT 180s group and the DC 75% CT 180s group.

The 25% duty cycle group had a higher number of white blood cell/high power field than the 75% duty cycle group, which was larger than the sham group (figure 5-5). There was a significant difference between the sham and 25% duty cycle group. However, the 75% duty cycle group was not significantly different from the 25% duty cycle or sham groups. Although the 180s cycle time group had the highest percent white blood cell/high power field, it was not significantly different than either the 20s cycle time or sham group (figure 5-6).

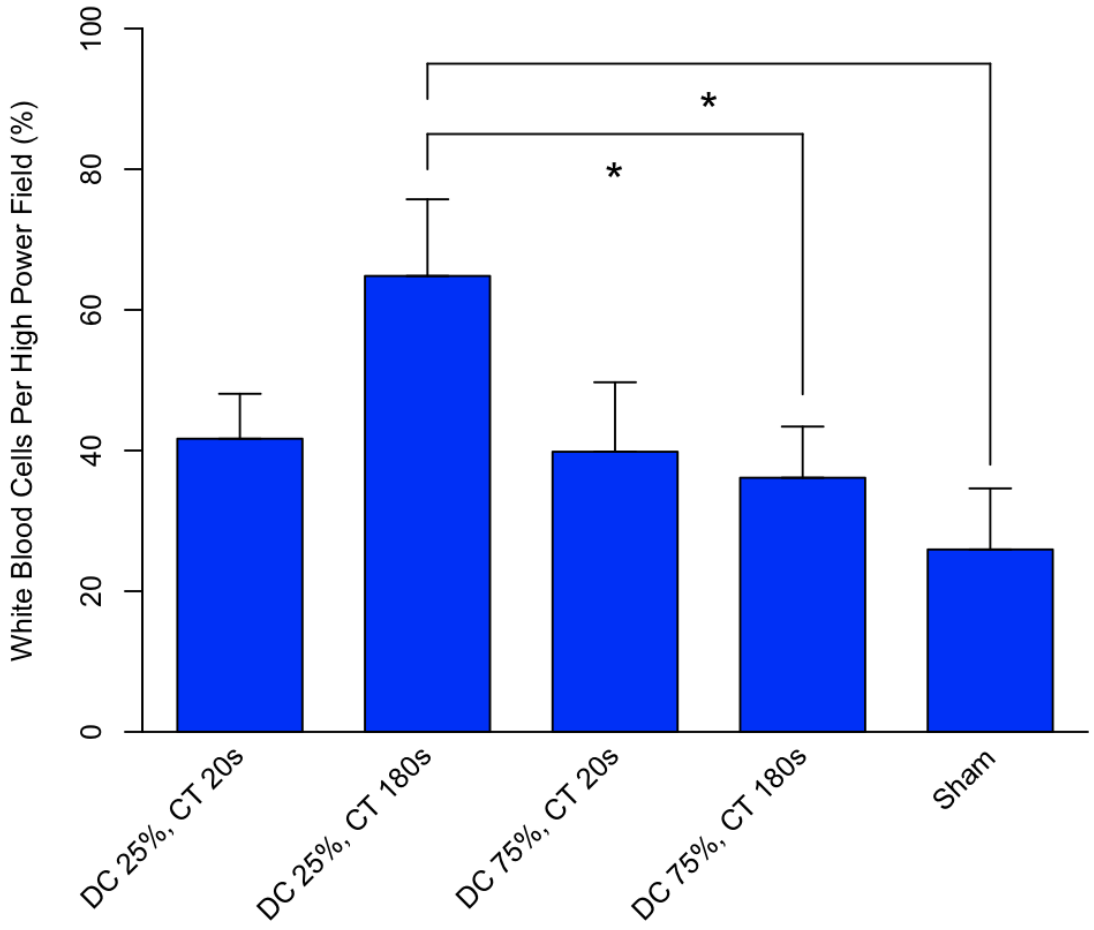


Figure 5-4: H&E comparison between work/rest groups for the 1-day experiment. Significant differences ($p<0.05$) are indicated by the brackets and the * symbol.

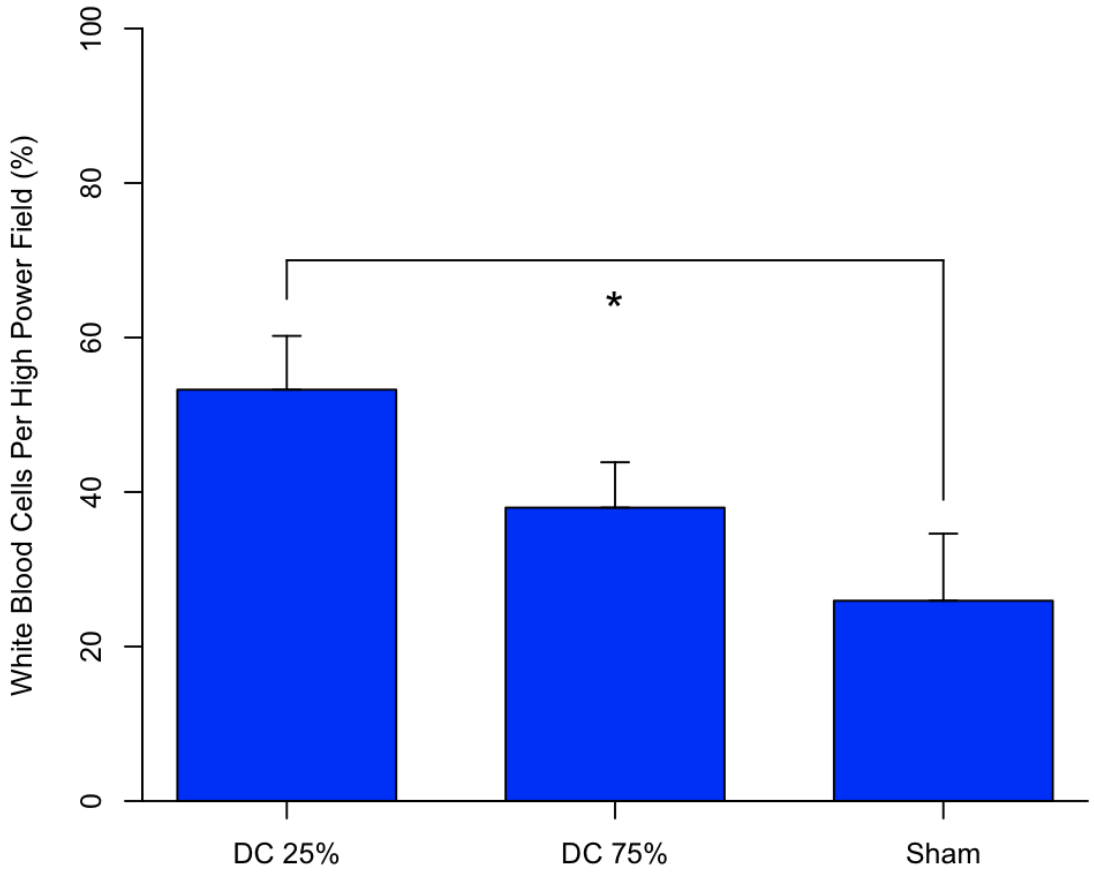


Figure 5-5: H&E comparison between duty cycle groups for the 1-day experiment. Significant differences ($p<0.05$) are indicated by the brackets and the * symbol.

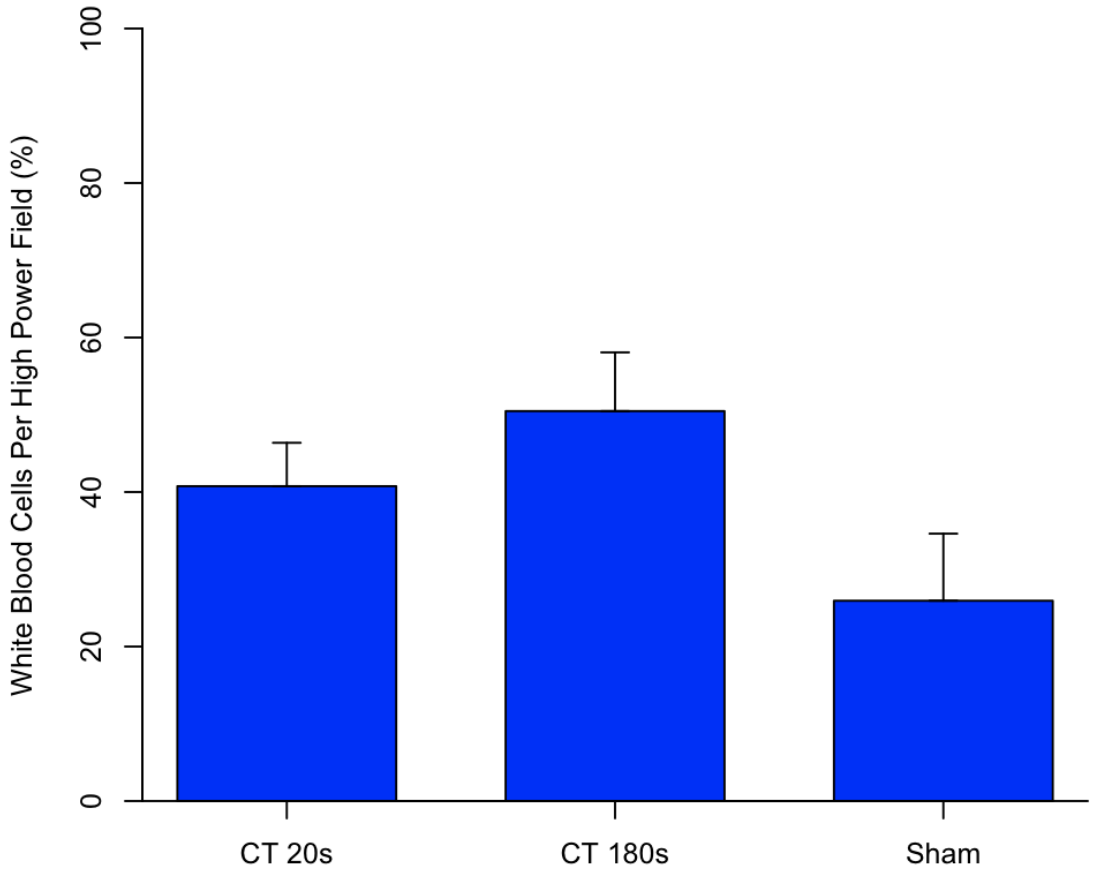


Figure 5-6: H&E comparison between cycle time groups for the 1-day experiment

5.3.3 H&E Analysis of the 3 Day Experiment

The sham group did not have the lowest percent white blood cell/high power field compared to the 4 work/rest groups (figure 5-7). In fact, only the 25% DC 20s CT group had a higher percentage white blood cell/high power field than the sham group. Both groups containing a 25% duty cycle contained a higher percentage of white blood cells/high power field than the 2 groups with a 75% duty cycle. None of the comparisons resulted in significant differences.

The comparisons between the duty cycle groups found the 25% duty cycle with a higher percent white blood cells/high power field than the 75% duty cycle group (figure 5-8). The sham group had a similar percentage of white blood cells/high power field as the 25% duty cycle group. The duty cycle group comparisons did not result in significant differences. The sham group had a higher percent white blood cells/high power field than the 180s cycle time group. None of the comparisons were significantly different (figure 5-9).

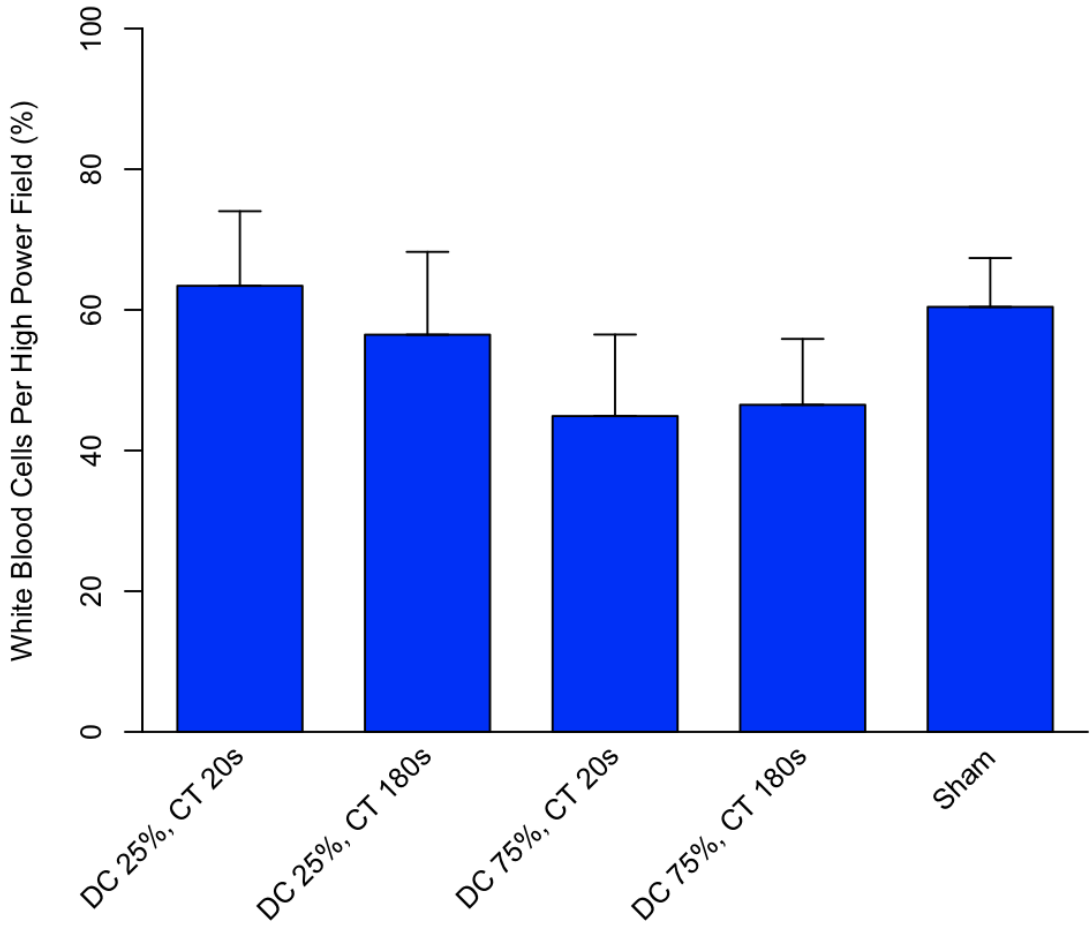


Figure 5-7: H&E comparison between work/rest groups for the 3-day experiment

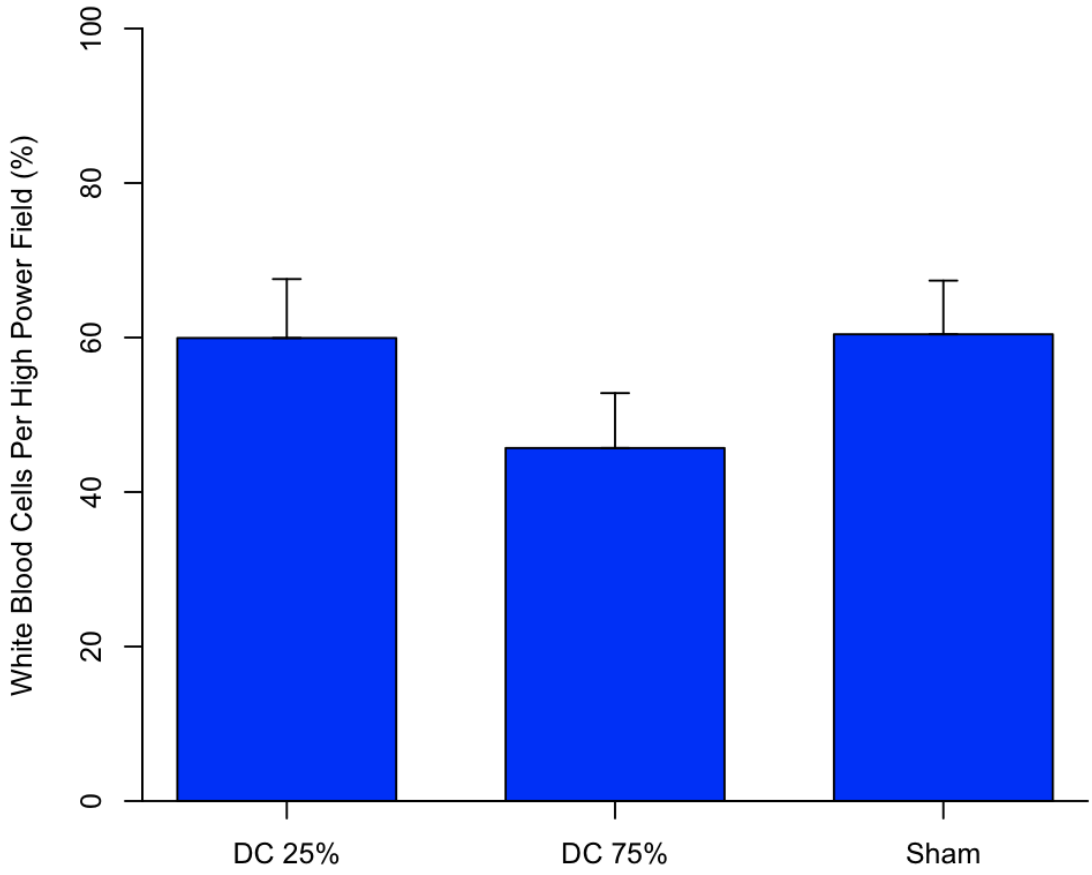


Figure 5-8: H&E comparison between duty cycle groups for the 3-day experiment

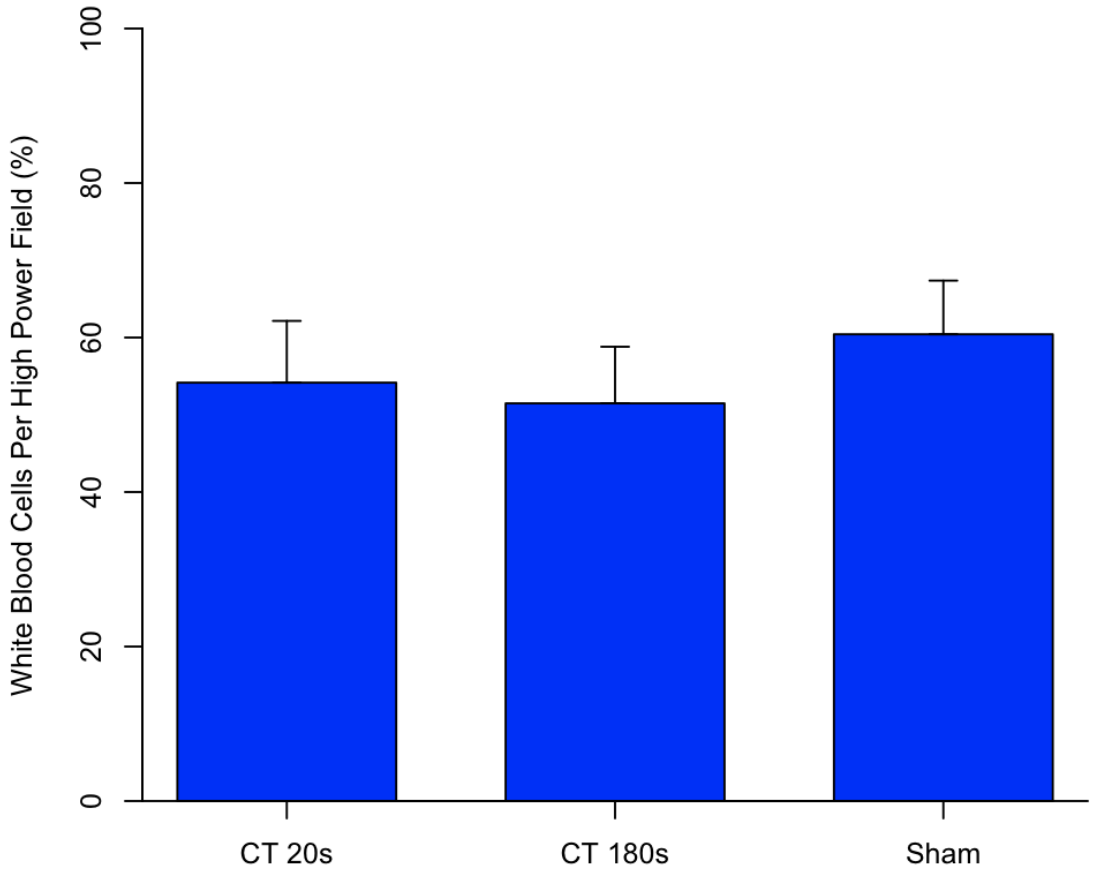


Figure 5-9: H&E comparison between cycle time groups for the 3-day experiment

5.3.4 H&E Comparison of the 1 & 3 Day Experiments

Overall, there was a significant difference in percent white blood cells/high power field between the 1 day and 3 day experiments (figure 5-10). The 3 day experiment work/rest groups all had a higher percent white blood cell/high power field than the 1 day experiment groups, except the 25% DC 180s CT groups (figure 5-11). The groups with the highest percent white blood cell/high power field were the 3 day 25% DC 20s CT and 1 day 25% DC 180s CT groups. Only

the comparison between the 1-day and 3 day sham groups resulted in significant differences. Both 3 day duty cycle and both 3 day cycle time groups had higher percent white blood cell/high power field than their respective 1 day groups (figures 5-12 and 5-13). None of the comparisons resulted in significant differences.

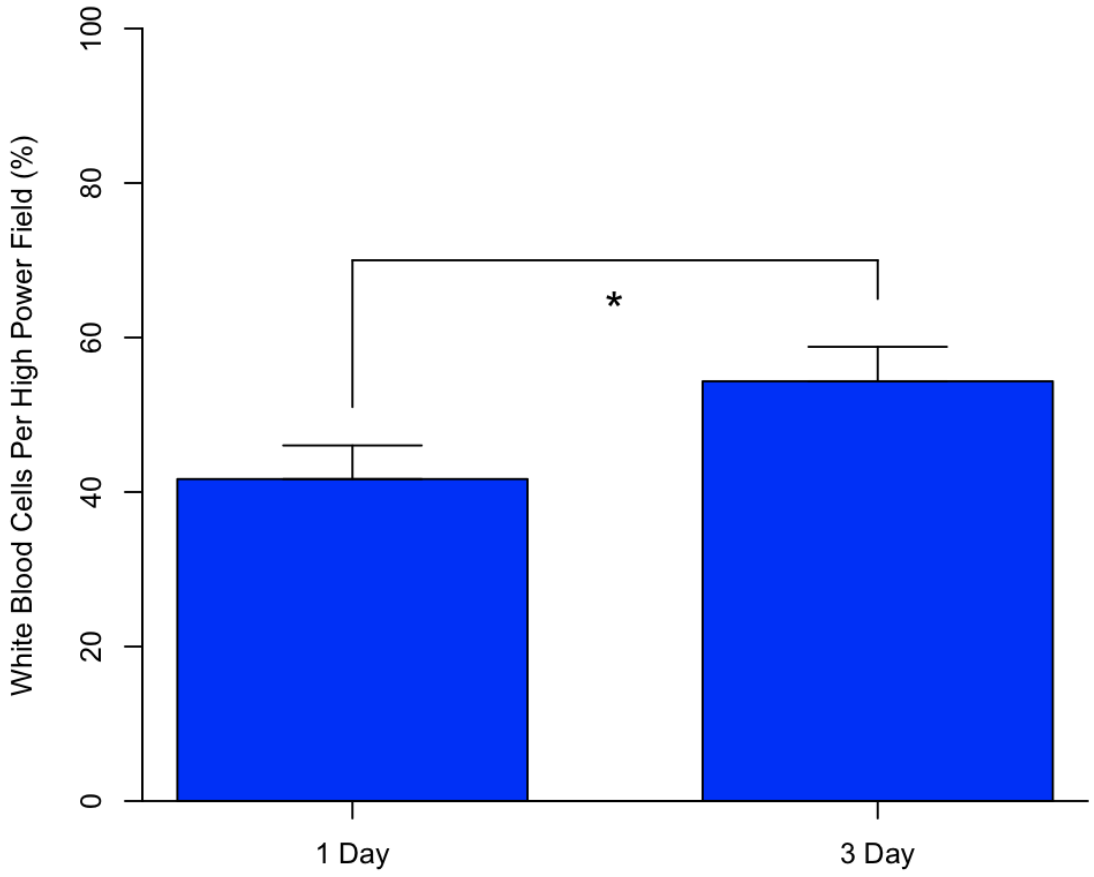


Figure 5-10: H&E comparison between the overall 1 and 3-day experiments. Significant differences ($p<0.05$) are indicated by the brackets and the * symbol.

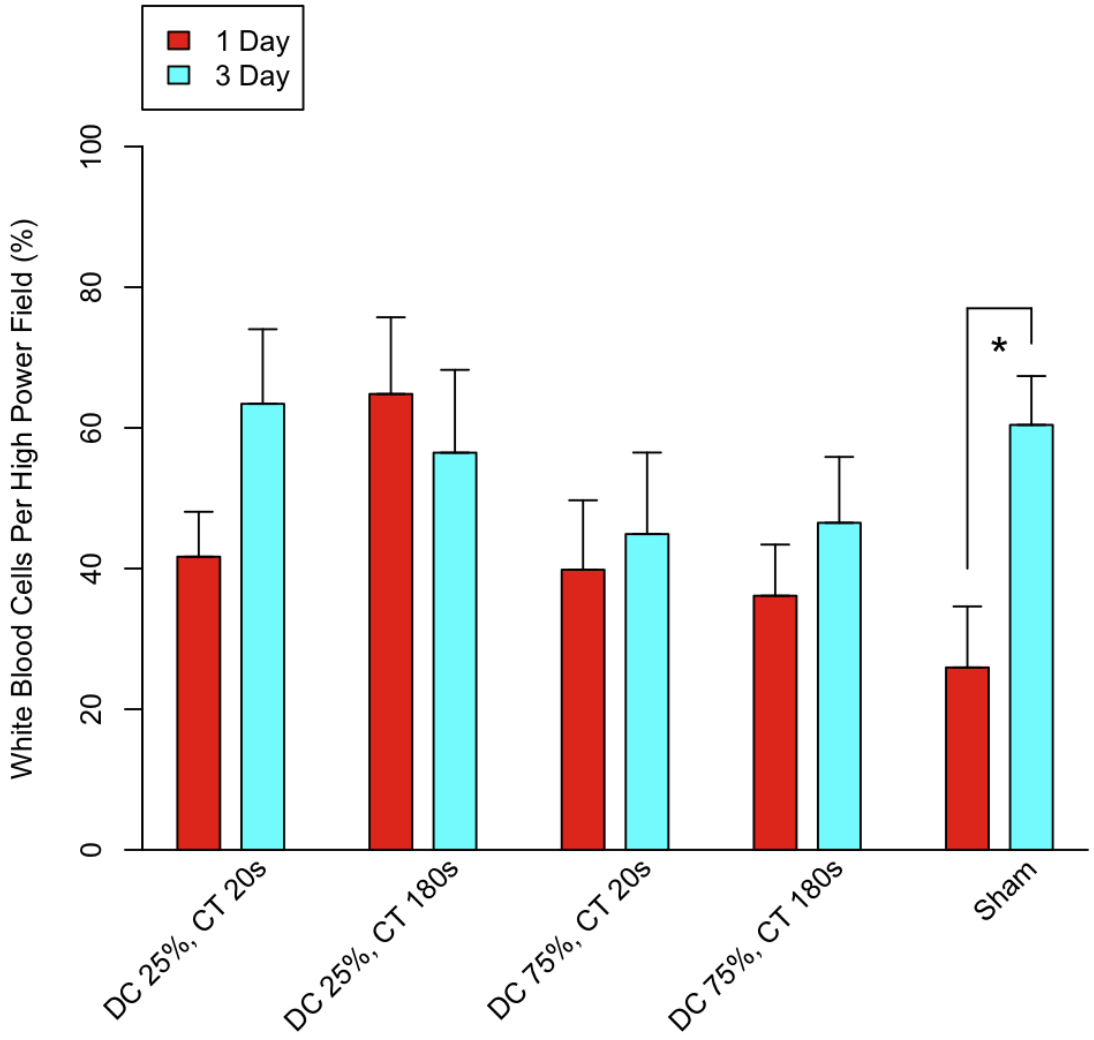


Figure 5-11: H&E comparison between work/rest groups for the 1 and 3 day experiments. Significant differences ($p<0.05$) are indicated by the brackets and the * symbol.

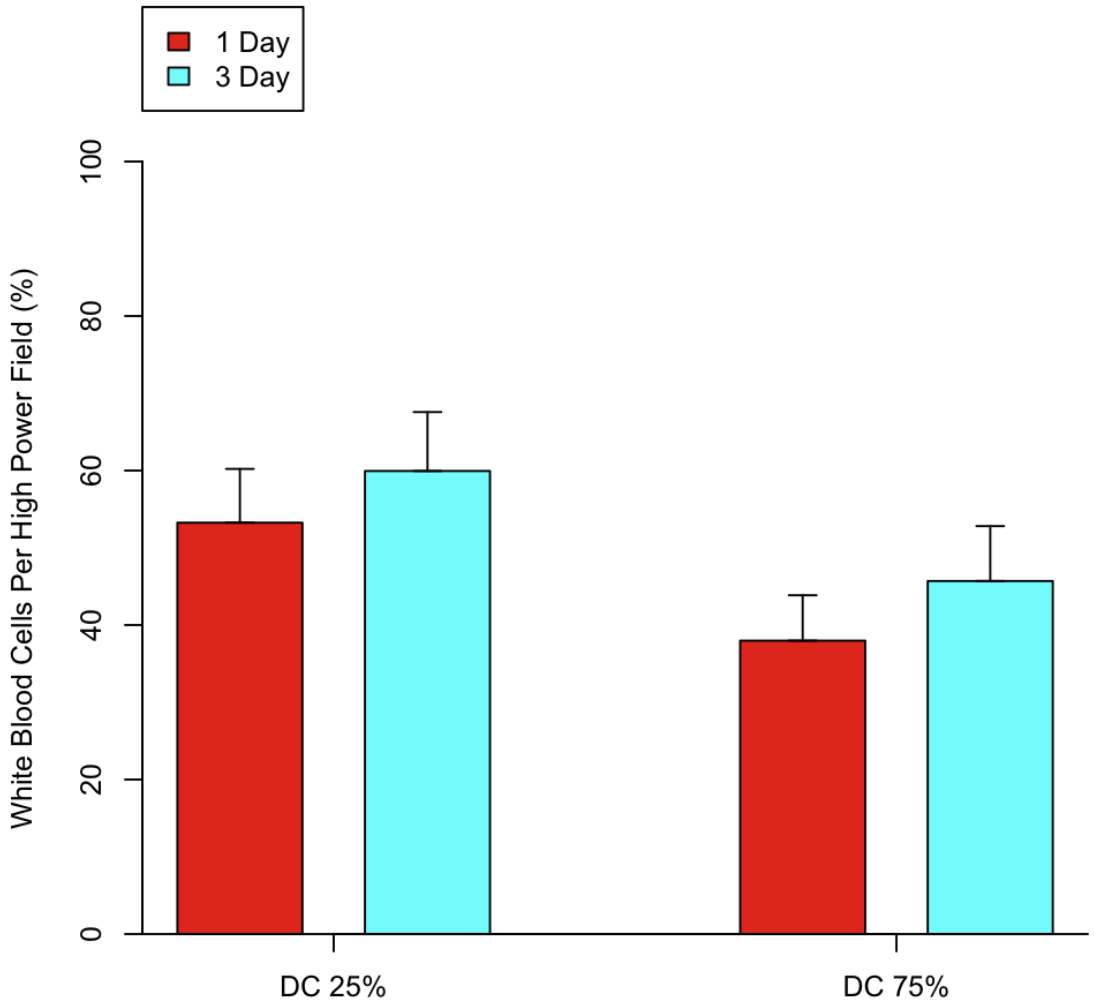


Figure 5-12: H&E comparison between duty cycle groups for the 1 and 3 day experiments

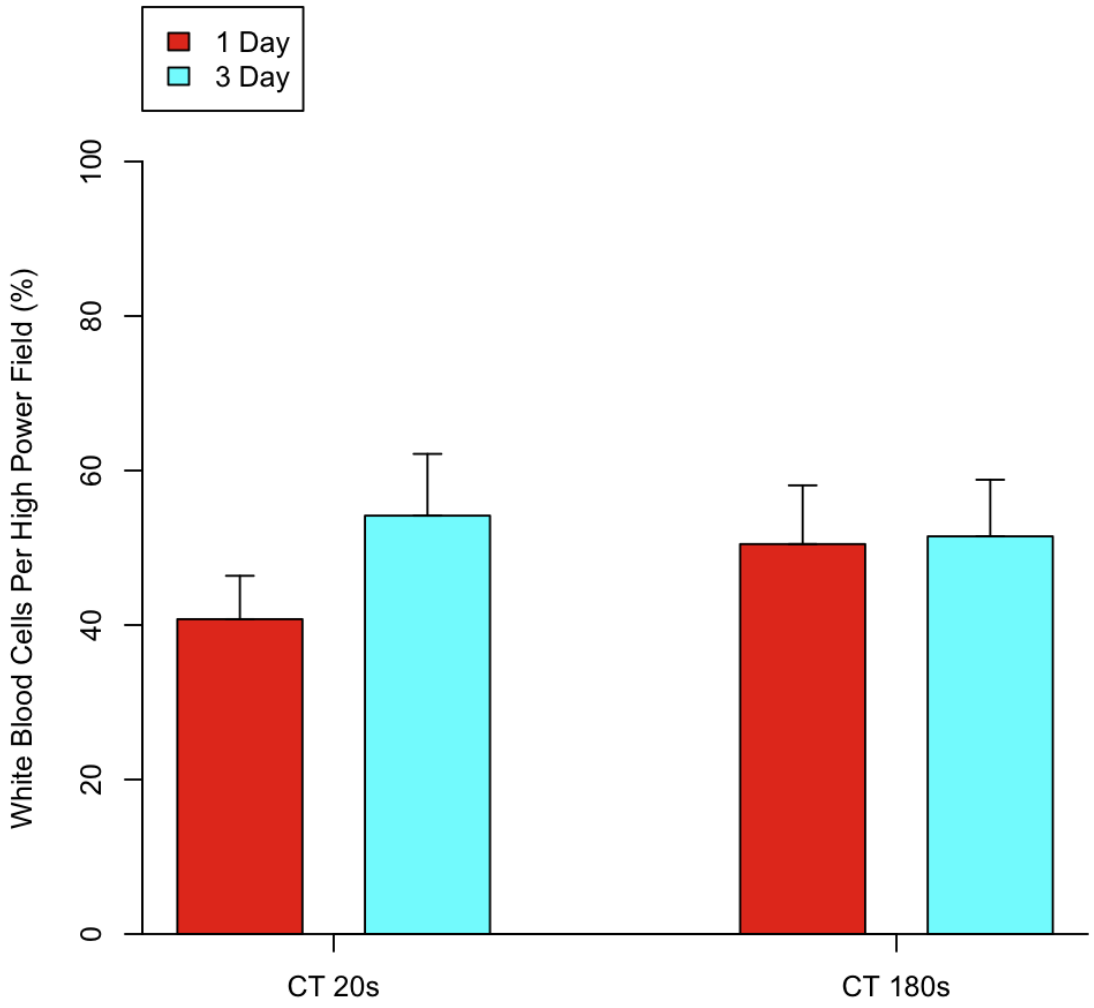


Figure 5-13: H&E comparison between duty cycle groups for the 1 and 3 day experiments

5.4 Discussion

The NADH fiber type analysis found that there was a higher percentage of fast twitch compared to slow twitch fibers. This result was in line with previous histological assessments of the rat medial longissimus muscle, where it was reported that fast twitch fibers were the dominant type (Schwartz-Giblin, 1983). These results agree with a study on lumbar muscles of the cat, in which they also found predominantly fast twitch muscle fibers (Carlson, 1978). The Schwartz-

Giblin (1983) and Carlson (1978) experiments analyzed several low back muscles, as well as several parts of these muscles, making a direct comparison to this study difficult. However, the general trend for all the studies found that the low back muscles contained a higher proportion of fast twitch muscles compared to slow twitch muscles.

The extent of muscle damage due to electrical stimulation was minimal, particularly in comparison to the positive control bupivacaine induced injured muscle. In that muscle there was a marked inflammatory response with numerous white blood cells, (including ED1 macrophages), moth-eaten fibers, structural damage as visualized by lack of desmin staining, and evidence of regeneration. Only the H&E stains provided evidence of injury in the experimental muscles and in these stains the number of white blood cells were few. The other stains did not indicate that damage occurred to the muscle fibers that is characteristic of these stains.

These results are similar to those found in Lexell et al., (1993). They stimulated rabbit hind legs 24 hours/day either continuously or intermittently over a period of 9 days. The amount of muscle injury reported was low and they found that less muscle injury with intermittent stimulation compared to the continuous stimulation.

Although there was a small number of white blood cells found, there was an expected trend in the 1-day experiment. The sham group had the lowest percent of white blood cell/high power field compared to all of the work/rest

groups. It was significantly lower than the 25% DC 180s CT groups. It was also significantly lower compared to the 25% DC group.

While the sham group was expected to have the lowest number of white blood cell/high power field it was somewhat surprising that the DC25% CT 180s work/rest group and the 25% duty cycle group had the highest occurrence of white blood cell/high power field. The 75% duty cycle represents the more taxing protocol and would assume to result in a higher injury response. A possible explanation may be that the DC 25% CT 180s work/rest group did not fatigue as much as the other groups, allowing the muscle fibers to participate more during the stimulation, causing an injury response. This group had the most rest allowance available, providing ample opportunity for recovery so that most, if not all, of the fibers would contract with each stimulation. The other groups may have had fibers too fatigued to be able to contract, thereby limiting the exposure to injury. The same argument can be said for the 25% duty cycle group. Further discussion on this topic will be provided in chapter 6.

The 3-day experiment did not result in any significant differences between the work/rest groups or between the duty cycle and cycle time groups. Unlike the 1-day experiment, the sham group did not contain the lowest percentage of white blood cell/high power field. The lack of differences between the groups may have been due to the additional days of having the electrodes in the medial longissimus muscles. In the 3-day experiment, the electrodes were surgically implanted 5 days prior to the start of the experimental protocol, whereas the 1-day experiment began immediately after the electrode implantation.

It was observed that upon harvesting the tissue it was more difficult in the 3-day experiment rats to remove the tissue and separate the electrodes compared to the 1-day experiment rats due to an excess of tissue growth around the electrodes. The electrodes may have been rubbing against the muscle while implanted, since the rats' movements were not restricted during the experimental time period. The rats in the 3-day experiment had more time with the electrodes implanted, which could have contributed to the additional tissue growth. The tissue growth appeared to be granulation tissue with associated collagen deposition.

A difference between the experimental rats and the bupivacaine control rats was that the inflammatory cells found in the experimental rats were only found outside of the muscle tissue, while the bupivacaine control rats had evidence of inflammation in both the extracellular space and within the muscle fiber. The similarity in number of white blood cells found in the sham rats compared to the experimental rats may have been due to the collagen deposition resulting from the rubbing of the electrodes on the muscle. These findings indicate that the increase in white blood cells may not have been a true inflammatory response due to the electrical stimulation, but due to the electrode implantation.

In support of this theory, a comparison between the 1 day and 3 day experiments found a significant difference in the percent white blood cell/high power field. Each work/rest group for the 3-day experiment, except the DC 25% CT 180s group had a higher percent white blood cell/high power field than the 1-

day experiment groups. The largest difference occurred between the sham groups and this was the only significant difference that occurred.

Thus, there was no significant effect between the 1 day and 3 day experiments for all the groups. There was an overall trend that the 3-day experiment had a higher inflammatory response. This was probably caused by the increased amount of time the electrodes were in the muscle for the 3-day experiment, as the sham groups provided a significant difference.

In summary, the various electrical stimulation work/rest protocols produced a small amount of injury. There were elevated white blood cells in the 1-day experiment work/rest groups compared to the sham group, supporting a basis for potential injury due to the stimulation protocols. However, there was little differentiation in white blood cell counts between the protocols to determine which work/rest ratio would be most detrimental to muscle. The 3-day experiment findings were elevated, even in the sham group, compared to the 1-day experiment, possibly due to the length of time the electrodes remained in the muscle.

CHAPTER 6 – CORRELATING FATIGUE AND INJURY BY STATISTICALLY ANALYZING M-WAVE AND HISTOLOGICAL RESULTS DUE TO *IN VIVO* ELECTRICAL STIMULATION OF THE MEDIAL LONGISSIMUS RAT MUSCLE

6.1 Introduction and Rationale

Calcium (Ca^{2+}) is the key to the relationship between muscle fatigue and injury. In order for a muscle to contract, Ca^{2+} must be released from the sarcoplasmic reticulum (SR). It is the goal of the neuromuscular transmission and excitation-contraction (E-C) coupling processes to contract muscle fibers by managing Ca^{2+} release and uptake. However, an increased amount of Ca^{2+} within the cell can lead to muscle damage.

Disruption at any of the steps in the release of Ca^{2+} can result in muscle fatigue. Fatigue then may serve as a protective mechanism to prevent an increased amount of Ca^{2+} release. For example, failure of neuromuscular transmission due to factors affecting the Na^{+}-K^{+} ATPase pump inhibits the signal from progressing toward the T-tubule, resulting in decreased force due to the lack of contraction. Prior to this failure, there may have been a high rate of electrical transmission leading to increases in Ca^{2+} release. By fatigue in the neuromuscular transmission further release of Ca^{2+} is inhibited, protecting the muscle fiber.

Electrically stimulating muscle results in a recorded compound action potential known as an M-wave. There is evidence to suggest that the M-wave is a good indicator of the neuromuscular transmission and the decrease in the M-wave signal a good indicator of neuromuscular transmission fatigue (Fowles et al., 2002). Although fatigue occurs due to electrical stimulation, the

neuromuscular transmission is not completely inhibited, as there can still be M-wave activity as evidenced in the current study at 6 hours of stimulation (figures 4-7 to 4-9). It was also found that certain electrical stimulation work/rest protocols inhibit the transmission less than others. For example, figures 4-16 to 4-18 indicate that there is recovery of the M-wave, and subsequently the neuromuscular transmission, between cycles. This recovery, if the stimulation is prolonged enough, may lead to increased Ca^{2+} release. If the duty cycles are high and cycle times short such that neuromuscular transmission recovers but reuptake of Ca^{2+} by the SR is not complete, an excess amount of intracellular Ca^{2+} may remain.

6.1.1 Specific Aim VII

Electrical muscle stimulation has provided evidence of fatigue (chapter 4) and injury (chapter 5). The purpose of this chapter was to determine if there was a relationship between fatigue and injury. Specifically, the data from chapters 4 and 5 were correlated to determine if there was a relationship between the change in M-wave signal and the evidence of percentage of white blood cells per high power field for the work/rest, duty cycle, and cycle time groups.

6.2 Methods

6.2.1 Statistical Analysis

The analyses were performed on a total of 48 Male Sprague-Dawley rats (400-450g), 24 in each of the 1-day and 3 day experiments. The percentage of white blood cells per high power field (H&E) for each rat was calculated for the 1-day and 3 day experiments. The M-wave data consisted of 6 separate calculations: the average change in the amplitude, area, and duration values

compared to the initial cycle and the average change in the amplitude, area, and duration values compared to the beginning of each cycle. For example, in order to obtain the average change between the cycles, for the 1-day experiment the values for each cycle was determined, averaged, and subtracted from the initial cycle for each rat. The 3-day experiment values used the percent change values at the end of each day to obtain the average change between cycles. The mean change from the beginning to the end of each cycle was calculated by averaging these values for each rat in the 1-day and 3 day experiments.

A correlation for each rat using the Pearson Product Correlation Test was then performed between the histology and M-wave data and categorized into the work/rest groups. Specifically, the mean change in M-wave values: amplitude, area, and duration, were correlated to the percentage of white blood cells per high power field in the 1 Day experiment and the 3 Day experiment, separately. The number of white blood cells per high power field was also correlated to the mean change in M-wave parameters (amplitude, area, duration) from the beginning to the end of each cycle. The work/rest groups were then separated into duty cycle and cycle time groups and the Pearson Product Correlation tests were repeated in these groups.

An alpha level of $p<0.05$ was considered significant for these analyses. All statistical procedures were performed in PASW Statistics v18.1 (SPSS, Inc Chicago, IL). The Pearson Product Correlation test was used due to the scalar data.

6.3 Results

6.3.1 Comparison Between Cycles

A plot of the average changes in amplitude, area, and duration for each rat over the length of the (1 day) 6-hour experiment compared to the percentage of white blood cells per high power field is provided in figure 6-1. Overall, the correlations were moderate to weak. Only the area comparison for group A provided a correlation above 0.8 and this was also the only correlation that was significant ($p<0.05$). This group had a percentage of white blood cells per high power field between 20 and 60 percent, with an area change ranging between approximately -0.0004 V-ms to 0.001 V-ms. It was positively correlated, meaning that as the M-wave increased in area, the percentage of white blood cells per high power field increased. Group B area in the 3-day experiment approached significance ($p<0.08$) with a -0.76 correlation (figure 6-2). M-waves were found to decrease in area with an increasing percentage of white blood cells per high power field, resulting in the negative correlation.

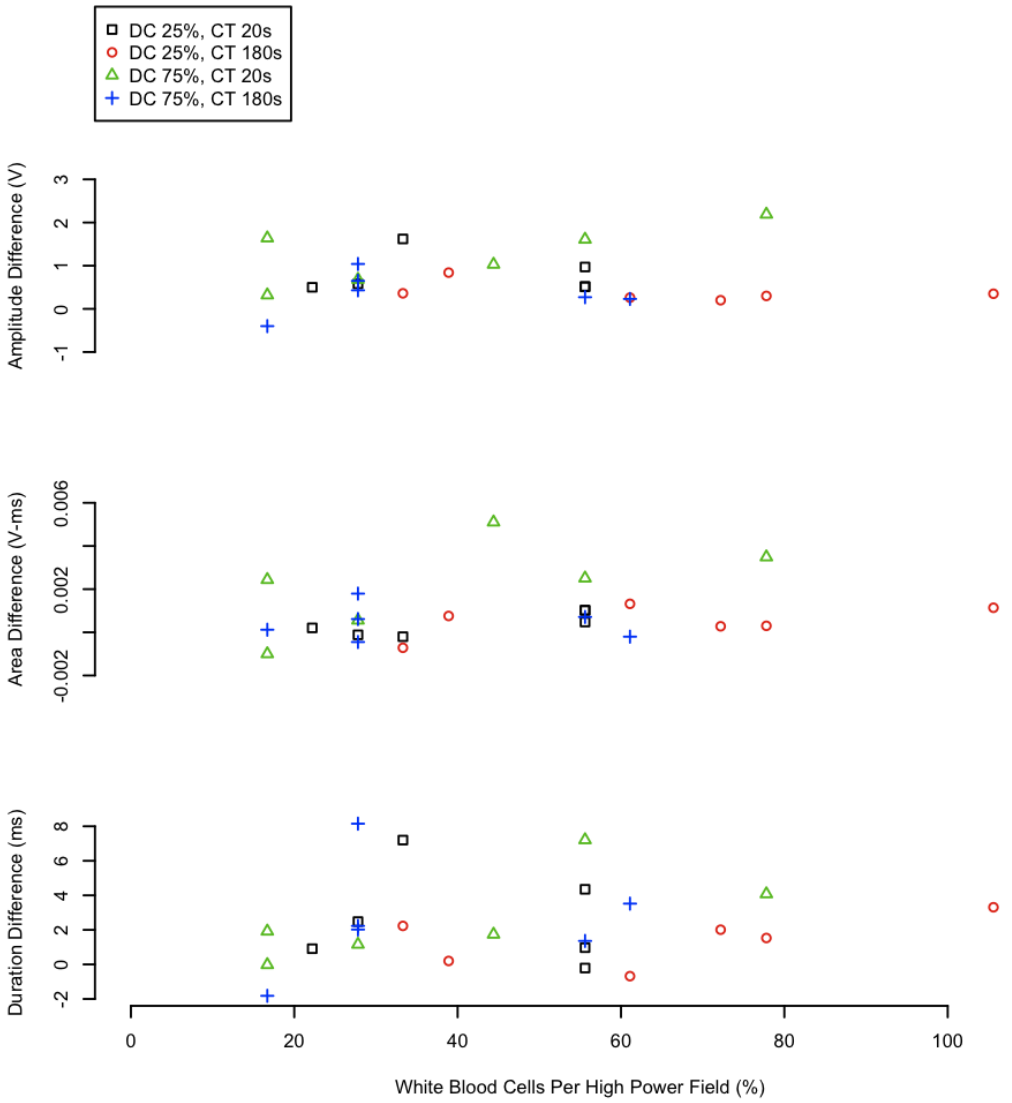


Figure 6-1: Correlation between the average difference for each cycle compared to the initial cycle in M-wave amplitude, area, and duration with histology data for the 1-day experiment

A comparison of the duty cycles and cycle times to the percentage of white blood cells per high power field did not result in any strong correlations, nor any significant correlations. The significant correlation found between area and percentage of white blood cells per high power field reported above may have been influenced by duty cycle. The 25% duty cycle comparison to percentage of

white blood cells per high power field approached significance ($p<0.06$) and was moderately strong (>0.5).

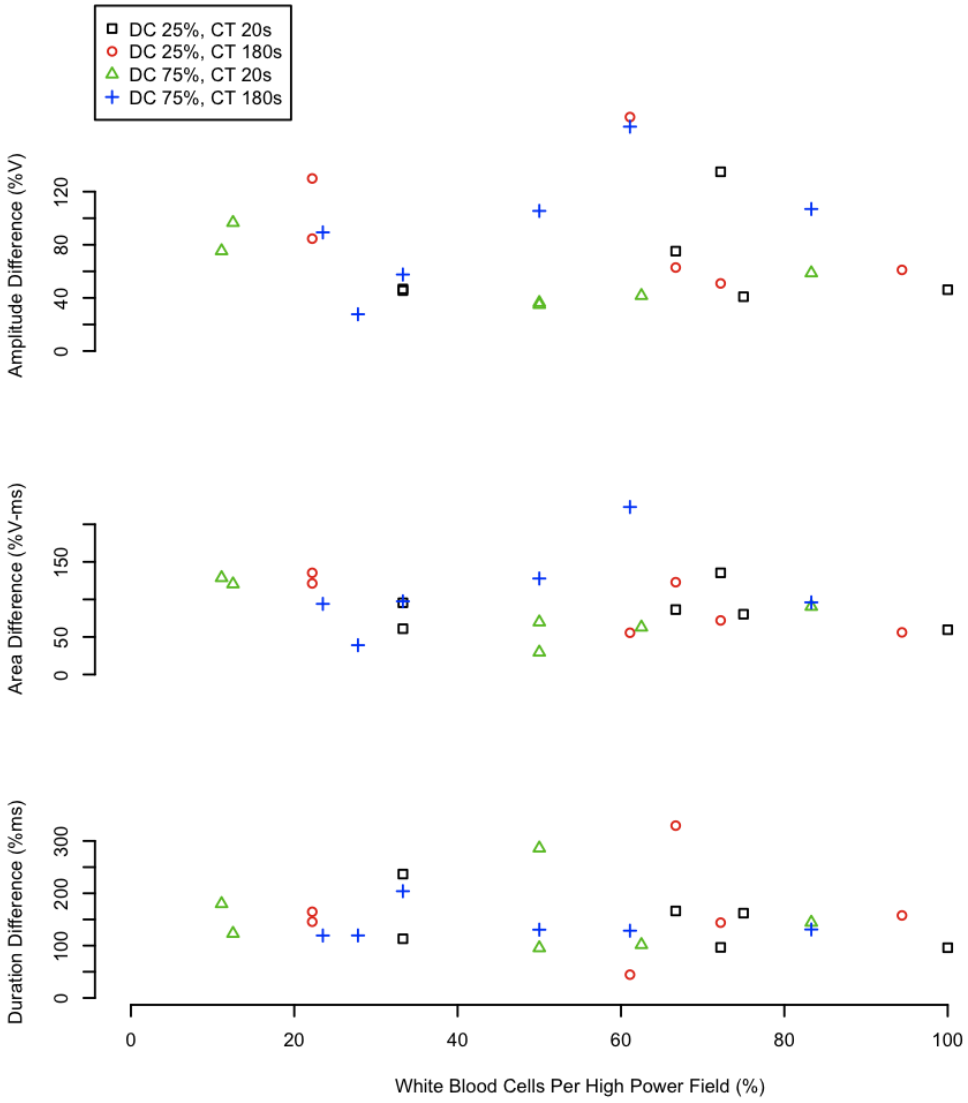


Figure 6-2: Correlation between the average difference for each cycle compared to each day's initial cycle in M-wave amplitude, area, and duration with histology data for the 3-day experiment

6.3.2 Comparison From the Beginning to End of Each Cycle

Figures 6-3 and 6-4 display the results of the comparisons between the average difference of amplitude, area, and duration of the M-wave signal from beginning to the end of each cycle for each rat, separated by group compared to the percentage of white blood cells per high power field in the 1 day and 3 day experiments, respectively. Out of all of the correlations made for each group and each M-wave measurement, only M-wave duration in group C for the 1 day experiment resulted in a high (0.8) and significant ($p<0.05$) correlation. The average change in M-wave duration for this group of rats was generally smaller than the other groups and they were grouped in the lower percentage of white blood cells per high power field. Correlations between duty cycle and cycle time and percentage of white blood cells per high power field were weak for each of the 1 day and 3 day experiments.

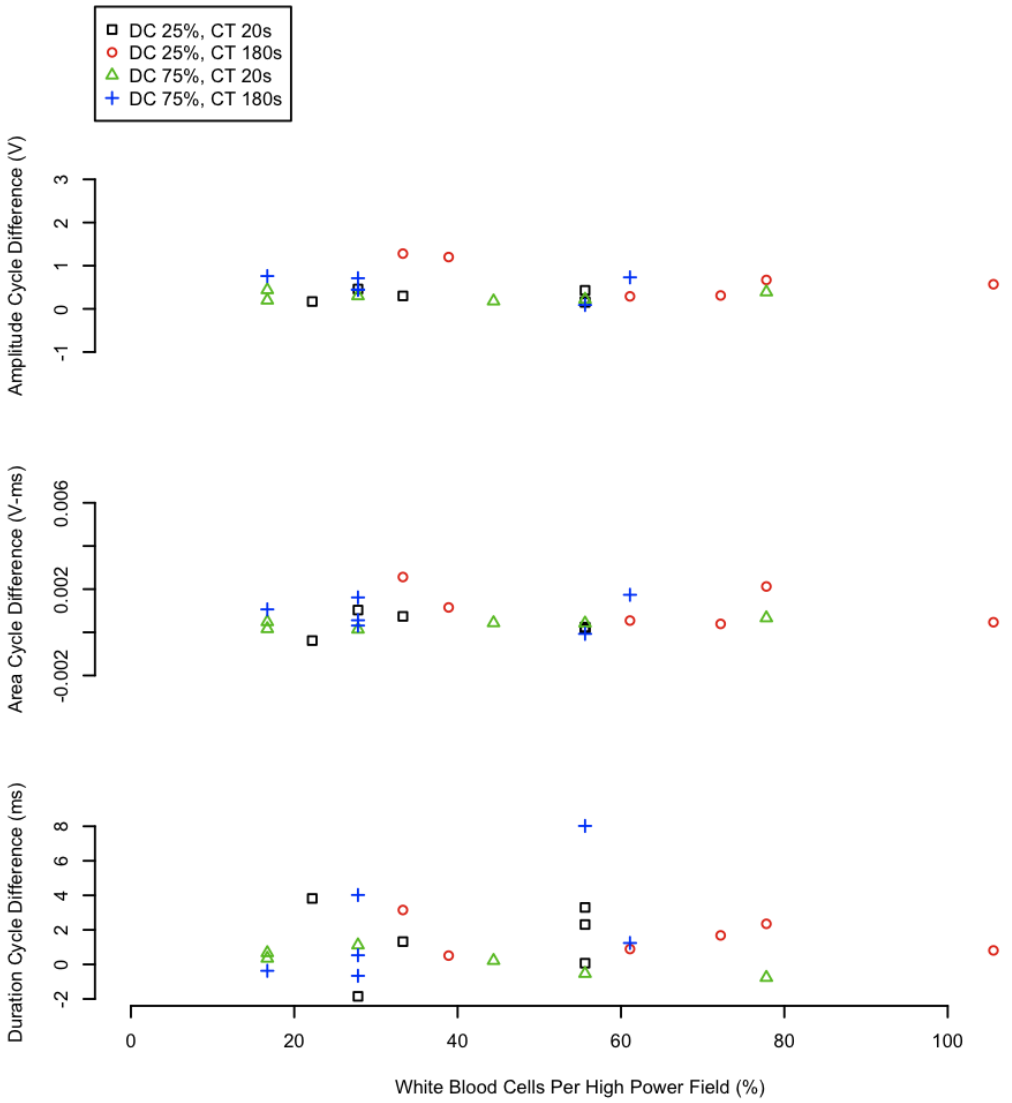


Figure 6-3: Correlation between the average difference from beginning to end of each cycle in M-wave amplitude, area, and duration with histology data for the 1 day experiment

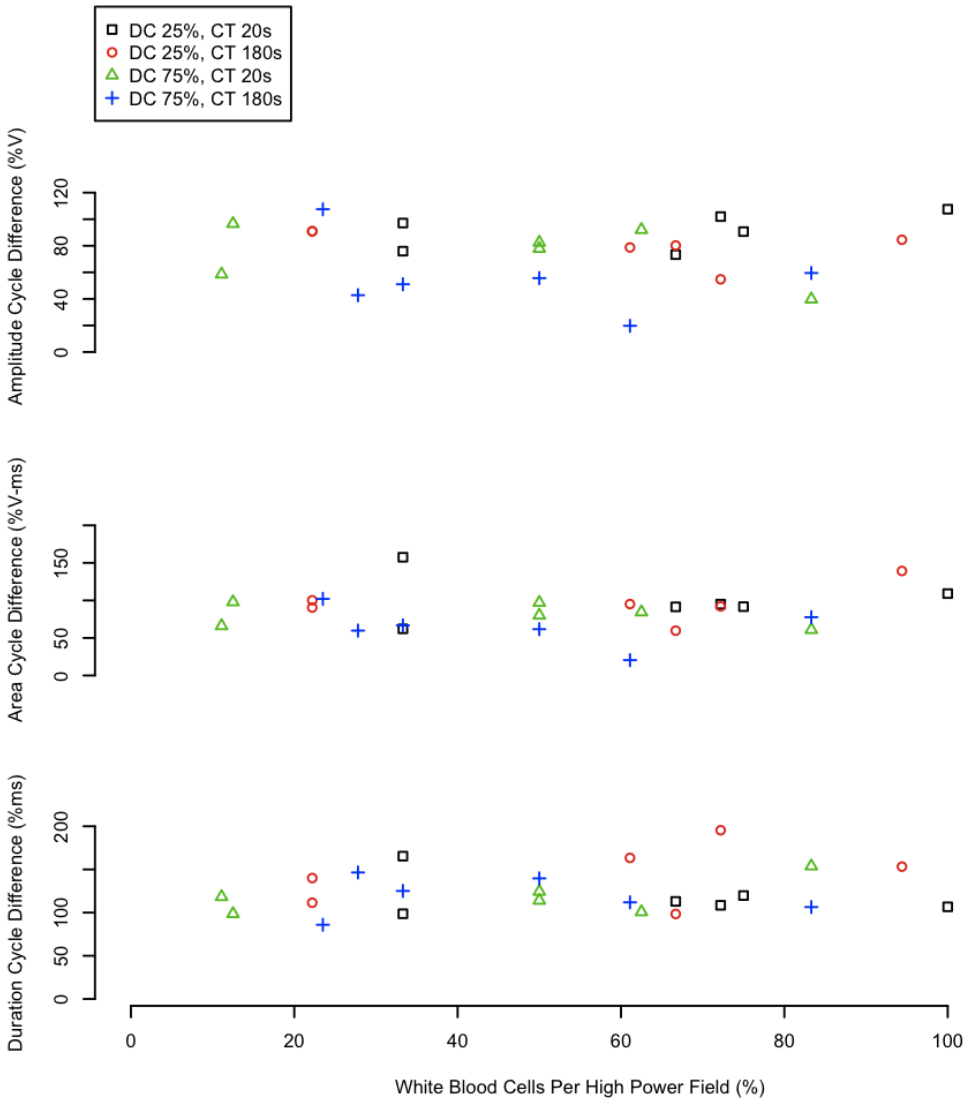


Figure 6-4: Correlation between the average difference from beginning to end of each cycle in M-wave amplitude, area, and duration with histology data for the 3 day experiment

6.4 Discussion

There was limited indication that electrophysiological evidence of muscle fatigue and histological evidence of injury was related, based on the correlations between M-wave and histology data. Most of the correlations were moderate to weak. The average change of M-wave area over the course of the 1-day experiment in group A was correlated with a low number of percentage of white

blood cells per high power field. This indicated that a low duty cycle (25%) and short cycle time (20s) resulted in little fatigue and injury. The group C (DC 75% CT 20s) average change in duration from the beginning to the end of each cycle in the 1 day experiment also had a high correlation with percentage of white blood cells per high power field. This indicated that a high duty cycle and short cycle time resulted in a small change in duration from the beginning to the end of each cycle, leading to a decreased indicator of muscle injury.

The lack of correlation between the M-wave and histology results may be related to the variability in the histology results stemming from the low percentage of white blood cells per high power field. Chapter 5 determined that there was little difference between the work/rest groups, duty cycle, and cycle time groups for the percentage of white blood cells per high power field, which was likely related to this variability. This was emphasized in figures 6-1 to 6-4, as the individual rat data was widely distributed for the percentage of white blood cells per high power field results. Thus, there was little differentiation between the groups in regards to histological outcome.

The stimulation protocol was diverse enough to provide differentiation in fatigue between each of the work/rest, duty cycle, and cycle time groups. However, it was not sufficient to cause histological evidence of injury. Increasing the voltage may have provided increased evidence of injury, but may have also fatigued each group to the point of lack of differentiation. An alternative solution would have been to introduce an eccentric contraction to the protocol. Eccentric contractions have been found to result in increased injury, particularly in fast

twitch muscle fibers, and not to have an effect on the excitation-contraction coupling mechanisms of fatigue. These types of contractions occur in the workplace and may contribute to low back muscle injury.

Therefore, although the results did not indicate a correlation between fatigue and injury, the lack of correlation may have been due to the experimental protocol. There was not enough evidence of injury to differentiate between the groups. Increasing the amount of muscle injury without affecting the fatigue differentiation between groups may provide a relationship between fatigue and injury.

CHAPTER 7 – CONCLUSIONS

The objective of this study was to determine the electrical muscle stimulation effects of varying duty cycles and cycle times on the fatigue and injury of the rat medial longissimus muscle. Rats were divided into 4 groups, based on 2 duty cycles and 2 cycle times: Group A (DC 25% CT 20s), Group B (DC 25% CT 180s), Group C (DC 75% CT 20s), and Group D (DC 75% CT 180s). The rats were further subdivided into the number of days that they were stimulated. They were either stimulated for 6 hours in 1 day or 2 hours for each of 3 days.

The analysis of the electrical stimulation was performed to assess fatigue due to the various protocols. Specifically, the M-waves were analyzed for changes in amplitude, area, and duration of the signal in relation to the various duty cycle and cycle time groups. Histology procedures were used to assess the histological evidence of injury after exposure to the chosen duty cycles and cycle times. The histological procedures included H&E, NADH, ED1, Desmin, and Vimentin stains.

Based on the results of the M-wave analysis, the following conclusions were drawn:

1 The technique of implanting stimulating and recording electrodes into the medial longissimus in vivo produced reliable stimulus artifact and M-wave data corresponding to previously published studies in other muscle groups for the 1-day experiment. This is the first known study using these techniques in low back muscles.

2 A long duty cycle and short cycle time (e.g. DC 75% CT 20s) resulted in the greatest amount of fatigue due to the short rest allowance.

3 Duty cycle had the largest influence on fatigue when comparing each cycle over time.

4 The change in M-wave measurements from the beginning to the end of each cycle was influenced more by cycle time than duty cycle. For example, long cycle time produced more fatigue from the beginning to the end of the cycle than a short cycle time, regardless of duty cycle.

5 Implanting electrodes over several days produced a diminished signal compared to the day of implantation, possibly due to healing and regenerating tissue around the electrodes. The results of the comparison between days for the 3-day experiment M-wave data may not have been reliable due to the diminished signal, but it provided meaningful data within the same day.

6 There was evidence of fatigue in the 3-day experiment with a decrease in amplitude and area and an increase in duration from the beginning to the end of each day.

7 A comparison between work/rest, duty cycle, and cycle time groups did not provide a clear understanding as to the variables affecting fatigue in the 3-day experiment. This was most likely due to the diminished M-wave signal.

8 A comparison of the M-wave signals from the beginning to the end of each cycle for the 3-day experiment revealed fatigue occurred due to decreasing amplitude and area and increasing duration.

9 The fatigue in the 3-day experiment from the beginning to the end of each cycle was due to duty cycle, rather than cycle time, as evidenced in the 1-day

experiment. A higher duty cycle resulted in greater fatigue than the lower duty cycle.

10 Comparing the 1 day and 3 day experiment M-wave data found relatively little difference in percent change in amplitude, area, and duration across cycles.

11 However, there was a consistently larger change in the M-wave measurements from the beginning to the end of each cycle for the 1-day experiment compared to the 3-day experiment. This may have been due to the diminished signal in the 3-day experiment having less potential change.

The evaluation of the histology data provided the following conclusions:

1 There was an approximately 2:1 ratio of fast twitch to slow twitch fibers found in the rat medial longissimus muscle, as determined by the NADH stain.

2 The intensity of the stimulation protocols did not result in histological evidence of injury in the muscles, as evidenced in the NADH, ED1, Desmin, and Vimentin stains.

3 H&E staining produced a small number of white blood cells that were counted for the purposes of comparing injury between groups. However, the numbers were greater than in the sham group, indicating that there may have been an effect of the stimulation protocols on muscle injury.

4 While there was large variability in the white blood cells per high power field counts, probably due to the small amount of produced injury. The sham group in the 1-day experiment had the smallest percentage of white blood cell per high power field.

5 The 3 day experiment sham group did not differ in white blood cell count per high power field compared to the other groups, indicating that the extended period of time the electrodes were in the muscle may have had an effect on the inflammatory response.

6 A comparison between the 1 day and 3 day experiments found the 3 day experiment results with higher percent white blood cell per high power field than the 1 day experiment, further suggesting an inflammatory response due to the length of time for electrode implantation.

7 Correlations between the M-wave and histology data were moderate to weak and did not provide a relationship between fatigue and injury. This may have been due to the limited amount of injury produced in the muscle.

In summary, this study has suggested that isometric contractions, as a result of electrical stimulation, produced fatigue as measured by M-wave amplitude and area decrement and an increase in M-wave duration. Activities performed with a high duty cycle and short cycle time produced the greatest amount of fatigue over time, suggesting that low duty cycles with long cycle times may be preferable. This type of work/rest ratio would provide the largest rest allowance.

From the beginning to the end of each cycle, a long cycle time fatigued the muscle greater within that cycle, but allowed a greater recovery of the neuromuscular transmission the following cycle. It may be suggested that this fatigue served as a protective mechanism from injury to the muscle, as there was

little evidence of injury in the histology assessments. Additional research utilizing protocols with a combination of electrical stimulation and eccentric contraction are required to further understand the relationship between fatigue and injury.

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ABSTRACT

LONGISSIMUS MUSCLE FATIGUE AND INJURY RESPONSE DUE TO ELECTRICAL STIMULATION WITH VARIED WORK/REST RATIOS

by

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The estimated yearly cost of lost-time work injuries and illnesses is $140 billion. The average cost of musculoskeletal disorders (MSDs) exceeds all other claims. These injuries persist in spite of ergonomic interventions addressing known risk factors. Work/rest ratios have not received a significant amount of attention, particularly in low back disorders, and it is hypothesized that the lack of adequate rest within a work cycle may contribute to muscle fatigue and ultimately injury. The aim of the current study is to determine the duty cycle and cycle time combinations that contribute to muscle fatigue and injury.

Stimulating and recording electrodes were surgically implanted into the medial longissimus muscle of a total of 58 Male Sprague-Dawley rats (400-450g). These rats were separated into 4 work/rest groups as well as a 1 day and 3 day experiment. Fatigue, based on decreased M-wave amplitude and area throughout the 1-day experiment was greatest in the work/rest group consisting of the highest duty cycle and shortest cycle time. This group was significantly different ($p<0.05$) than the group exposed to the lowest duty cycle and longest

cycle time. Fatigue due to increased M-wave duration was observed in the group with the highest duty cycle and longest cycle time. Higher duty cycles had the largest effect on fatigue over the duration of the experiment, while longer cycle times were implicated in fatigue from the beginning to the end of each cycle. Fatigue in the 3 day experiment occurred from the beginning to the end of each day as well as from the beginning to the end of each cycle. Comparison between days was not reliable due to potential obstruction of the M-wave signal due to tissue healing around the electrodes.

The assessment of injury was performed through histological, histochemical, and immunohistochemical stains. Neither muscle injury nor regeneration was detected through NADH, ED1, desmin, and vimentin stains. The H&E reaction revealed a small percentage of increased inflammatory cell activity compared to the sham rats for the 1-day experiment. The low duty cycle with long cycle time group had a significantly higher white blood cell count compared to the high duty cycle and long cycle time. The 3-day experiment resulted in an overall elevated white blood cell count, including the sham group, compared to the 1-day experiment. There was little correlation between the M-wave fatigue data and the histology injury data. Overall, results from this experiment provide insight into muscle fatigue and injury due to various work/rest ratios.

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Publications

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