EFFECTS OF PHYTOECDYSTEROIDS ON SKELETAL MUSCLE CONTRACTILE FUNCTION AND PROTEIN SYNTHESIS AFTER ECCENTRIC MUSCLE DAMAGE

A Thesis
by
TYLER THOMAS RICE

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APPROVED BY:

R. Andrew Shanely, Ph.D.
Chairperson, Thesis Committee

Kevin A. Zwetsloot, Ph.D.
Member, Thesis Committee

Alan C. Utter, Ph.D., M.P.H.
Member, Thesis Committee

Kelly J. Cole, Ph.D.
Chairperson, Department of Health and Exercise Science

Max C. Poole, Ph.D.
Dean, Cratis D. Williams School of Graduate Studies

Abstract

EFFECTS OF PHYTOECDYSTEROIDS ON SKELETAL MUSCLE CONTRACTILE FUNCTION AND PROTEIN SYNTHESIS AFTER ECCENTRIC MUSCLE DAMAGE

Tyler Thomas Rice
B.S., Slippery Rock University
M.S., Appalachian State University

Chairperson: R. Andrew Shanely, Ph.D.

Our preliminary data demonstrate that phytoecdysteroids, such as 20-hydroxyecdysone (20E), have no effect on muscle hypertrophy in young sedentary mice. Yet in old sedentary mice we have shown an increase in protein synthesis signaling and muscle fiber size. These divergent observations suggest that phytoecdysteroids in young skeletal muscle may require a stimulus to be effective. The objective of this study was to determine if 20E enhances recovery of maximal tetanic torque production and additively stimulates Akt/mTOR signaling after 150 injurious eccentric skeletal muscle contractions (EC). Male C57BL6 mice (3-6 mo-old) were randomly assigned to either the EC + placebo or EC + 20E group. *In vivo* isometric contractions (Aurora Scientific, 1300A) were performed to obtain optimal electrode placement and voltage. Following electrode placement, *in vivo* torque frequency was assessed, 150 eccentric contractions were performed and *in vivo* torque frequency was administrated to assess torque production immediately post-injury to the anterior crural skeletal muscles. Upon completion of the EC and torque frequencies, the mice received an oral gavage of either 20E (50 $mg \cdot kg^{-1}$ BW) in liquid diet (BioServ AIN-76) or

placebo (liquid diet only) and allowed to recover. Mice were gavaged daily for 2 or 6 days. On day 3 or 7, post-injury torque frequency was assessed and skeletal muscles were harvested and prepared for assessment of Akt/mTOR signaling via Western blot analysis. No significant differences in recovery of torque production within groups were observed at any time point or treatment. Furthermore, no significant differences were found in activation of Akt, 4E-BP1, or rpS6 intracellular signaling between treatments. These data suggest that 20E does not additively stimulate an increase of protein synthesis and enhance recovery of torque production after eccentric contraction injury. Additional research may be warranted on the dosage amount that may additively increase protein synthesis and torque production after eccentric contraction injury.

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Dedication

First, to my thesis chairperson, Dr. R. Andrew Shanely, thank you for all your guidance and support throughout not only my time conducting research, but also with course work at Appalachian State University. You are and will continue to be a great mentor who I can look up to for support, a teacher, and a friend. You've taught me more than I would ever have imagined, considering I had little to no experience working with animals or wet lab experience. I will always carry with me all the valuable life advice you have shared with me while in the lab.

Second, to my thesis committee members, Dr. Kevin A. Zwetsloot and Dr. Alan C. Utter, thank you for you guidance and attention to detail throughout this process. Dr. Zwetsloot, I am very grateful for the all the things you have taught me, whether it be in the biochemistry lab with Western blots or working with the mice in the vivarium. These skills you taught me will be very valuable in the pursuit of a research career. Dr. Utter, I want to express how grateful I am to have had the overwhelming support and words of encouragement when it was needed most. Also, your attention to detail was much needed as an outside look in on this project.

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Foreword

This thesis will be submitted to a journal within the American Physiological Society. This thesis is formatted according to the style for the American Physiological Society.

Chapter 1: Introduction

Skeletal muscle is the most abundant tissue in the human body by mass and is susceptible to multiple types of injuries, for example, injury from eccentric contractions. Eccentric contractions occur when a strain is put on the muscle by which opposite tensile forces are present at the same time (11). The cost of injuries due to eccentric damage within hospitals is difficult to determine since hospitalization is typically not required. Without correct medical care a significant skeletal muscle injury can cause incomplete functional recovery and possibly reinjury (50). Currently there is no ideal immediate treatment for eccentric muscle injury but the most advised treatment is the use of the RICE (rest, ice, compress, and elevate) method (41).

Muscle injuries can lead to disrupted connective tissues, myofiber necrosis, hematoma, and inflammation. This disruption in muscle homeostasis can cause a loss of skeletal muscle strength as well as atrophying of the muscle leading to pain with movement, decreased range of motion, or general soreness (60). Few studies have sought ways to remedy muscle injuries. Nonsteroidal medications are typically used to decrease inflammation in injured skeletal muscle (55). Alternative ways of treating muscle injuries using anabolic agents as well as catabolic agents have been examined. Acutely, catabolic agents return force-generating capacity in muscles after injury (10). Chronically, anabolic agents recover force-generating capacity and morphology in muscle after injury, indicating that anabolic agents are worth further study on recovery of force-generating capacity and morphology after a muscle injury (10).

Phytoecdysteroids are being investigated to determine the anabolic effects on skeletal muscle. Phytoecdysteroids, which are derivatives of an arthropod steroid hormone called

ecdysteroids, are structurally related to cholesterol-based mammalian steroid hormones without the harmful side effects associated with anabolic steroids. Phytoecdysteroids work through a cascade of cellular mechanisms involving calcium flux into the cell and eventually cause activation of Akt (Protein Kinase B). Thus protein synthesis is maybe increased through supplementation with the phytoecdysteroids (32). Ecdysteroids increase protein synthesis in culture of muscle cells and increase force-generating capacity in rats (24, 32). The ecdysteroid 20-Hydroxyecdysone (20E) has also been shown to increase muscle fiber size (64). However, no studies have been conducted to determine if phytoecdysteroids will enhance the recovery of muscle function and morphology after eccentric contraction muscle injury. Therefore, the purpose of this study was to determine the degree to which phytoecdysteroids (1) enhance recovery of maximal tetanic torque production and (2) additively increase protein synthesis after a bout of eccentric contraction muscle injury. I hypothesized that phytoecdysteroids would (1) accelerate the rate of recovery of muscle maximal tetanic torque production and (2) additively increase the rate of protein synthesis after an eccentric contraction muscle injury to the anterior crural muscles compared to injured mice not receiving phytoecdysteroid treatment.

Chapter 2: Review of Literature

The purpose of this literature review is to provide background knowledge on how phytoecdysteroids function in the body of mammals and their ability to promote muscle growth through possible anabolic mechanisms. Furthermore, it is to provide knowledge on how eccentric contraction muscle injury models are utilized to simulate injuries and can be used in studies that are interested in investigating regeneration of skeletal muscle function. While studies have investigated these two ideas separately, there is a lack of knowledge on how phytoecdysteroids would mediate the muscle healing process after a muscle injury.

Phytoecdysteroids

Structure and Function

Phytoecdysteroids are plant derived analogues of ecdysteroids which are insect molting hormones. Structurally, phytoecdysteroids are cholesterol derived steroid hormones and are distinctly different than mammalian steroid hormones. Phytoecdysteroids are considered ketosteroids that have long carbon side chains. Phytoecdysteroids comprise a class of steroids with a polyhydroxylated cyclopentano perhydrophenanthrene ring system and are typically comprised of a 27-29 carbon skeleton. These polyhydroxylated ketosteroids are primarily produced by insects and plants (7, 31, 32, 39). The purpose of ecdysteroids that are produced within insects is to regulate metamorphosis, and several other pivotal life-cycle events. In plants, phytoecdysteroids serve as a chemical defense against insects or soil nematodes by disrupting the hormonal balance and molting processes. This chemical defense causes development disruption and can even cause eventual death in insects and soil nematodes (12, 45).

The most commonly studied phytoecdysteroid that has been studied is 20-Hydroxyecdysone (20E). 20E is reported to be safe when consumed by mammals due to its

low acute toxicity (25, 39). 20E is considered to exhibit very low acute toxicity in mice and rabbits when administered orally (7, 52). Phytoecdysteroid extracts (such as *Ajuga turkestanica extract)* have not shown any adverse side effects with exogenous supplementation such as atrophy of the testes or enlargement of tissues other than skeletal muscle (7, 39). A recent study has confirmed that 20E does not affect organ (heart, liver, spleen, kidneys, and testes) wet mass compared to body mass (47). Low acute toxicity in mammals indicates that phytoecdysteroids might not cause side effects or negative health risks compared to exogenous testosterone or estrogen supplementation. While bioavailable in the blood, low levels of the phytoecdysteroid 20E does not have any acute toxicity effects, but if administered over the LD_{50} (Lethal dose able to kill 50% of the exposed population) of 6g/kg it can cause high levels of toxicity possibly leading to eventual death in mammals (7, 26).

Sources

Phytoecdysteroids have been measured in over 100 terrestrial plant families that include plants such as ferns, gymnosperm and angiosperms as well as being found in both annual and perennial plants. Phytoecdysteroids have been found in plant species such as *Ajuga, Serratula, Silene and Leuzea* (7, 25). Some commonly eaten foods that contain phytoecdysteroids in noticeable amounts are spinach, quinoa, yams, and chestnuts (8, 33, 58). These have variable amounts of phytoecdysteroids, for example, quinoa has 4-12 times more 20E by dry weight when compared to spinach leaves (46). Very little of the world's plant life has been investigated to determine exactly how many and what type of plants actually contain phytoecdysteroids.

Protein Synthesis and Signaling Pathways

Phytoecdysteroids, including 20E, have profound impacts on metabolic function in mammals, specifically 20E. Within C57BL/6 mice the triceps brachii muscle mass increased after continuous infusion of 20E for five days, however the muscle mass of several other muscles were unchanged (20). Cheng et al. (21) also measured other physiological parameters but didn't observe a significant change; suggesting that 20E has mild local anabolic effects. Other studies have found that 20E may actually have potent anabolic properties. Treatment with 20E in rat hind limb muscles increases muscle cross sectional area and affects muscles differently (64). Toth et al. (64) suggest that 20E works in a muscle specific fashion rather than a muscle fiber dependent manner.

In C2C12 myotubes protein synthesis increased up to 120% compared to the control group starting at two hours, peaked at eight hours, and remained significant for the remainder of the 24 hour experiment (32). Another study confirmed the findings that 20E supplementation increased protein synthesis in C2C12 in a dose dependent manner (21).

Other metabolic functions due to supplementation of phytoecdysteroids have been measured. For example, phytoecdysteroids can have effects on lipid metabolism by causing hypocholesterolamic effects (26). This could be attributed to the conversion of cholesterol into bile acids (26). Also, phytoecdysteroids may decrease hyperglycemia which otherwise would be induced by administration of glucagon or by destruction of the pancreatic islet β-cells (26). The same study also suggests that phytoecdysteroids have effects on other organs, for example, liver, kidneys, skin and brain. The liver is caused to secrete more bile after supplementation with phytoecdysteroids. 20E supplementation can restore glomerular filtration rate (26). Phytoecdysteroids have also been reported to accelerate the healing

process of small wounds or burns on the skin (26). Further 20E supplementation protects neurons within the CNS against deleterious effects of various drugs (26). Overall, the above information suggests that there are possible benefits of supplementing with phytoecdysteroids in mammals with very few, if any, side effects.

Phytoecdysteroids Effects on Skeletal Muscle

Recent studies indicate that phytoecdysteroids, unlike mammalian steroids, utilize an androgen independent receptor (7, 32). These studies suggest that 20E works through a G-protein coupled receptor (31). This receptor when activated then activates phospholipase C (PLC). PLC breaks down phosphoinisotol 3-phosphate (PIP3) into inositol 3-phosphate (IP3). IP3 then travels to the sarcoplasmic reticulum (SR) and binds to the IP3 receptor (IP3R). Once IP3R is activated by IP3, intracellular calcium is released from the SR into the cytoplasm. The increase in intracellular calcium allows for activation of phosphatidylinositol-3-phosphate kinase (PI3K). PI3K produces the earlier mentioned PIP3. PIP3 then activates phosphatidylinositol dependent kinase (PDK1) as well as Akt (Protein Kinase B). Akt is a phosphorylated version of serine/threonine-specific protein kinases which is activated or phosphorylated by PI3K. Akt then phosphorylates and activates mammalian target of rapamycin (mTOR), and subsequently increases protein synthesis in skeletal muscle tissue. Akt can also decrease protein degradation through phosphorylation of Forkhead Box-O (FOXO) protein. Once FOXO is phosphorylated it is unable to translocate to the nucleus of the cell and increase the expression of key degradation proteins, Murf-1 and Mafbx. Ultimately, phytoecdysteroids in this context would increase protein synthesis (31, 32) and perhaps decrease protein degradation. Gorelick-Feldman et al. (32) observed an increased strength in rat grip strength after supplementation of 20E (50 mg/kg BW) for 28 days,

suggesting that 20E does indeed activate Akt phosphorylation. Another study by Lawrence (47) observed that when older mice (20·months old) are supplemented with 20E (50 mg/kg BW) for 28 days there was an increase in muscle fiber cross sectional area of the plantaris and triceps brachii. According to the previous studies one would think that supplementation of 20E would increase cross sectional area of muscle due to the increased activity on Akt phosphorylation at any time point. On the contrary, a recent study has shown that 20E does not acutely activate Akt phosphorylation or mTOR signaling in young healthy rats (1). This study suggests that a loading phase of 20E may be required to have any effects on Akt phosphorylation or the mTOR pathway. The combination of aforementioned studies suggests that phytoecdysteroids work on activation of Akt and the mTOR pathway, but how effectively they work remains unknown.

Concentrated extracts of phytoecdysteroids from plants such as *Ajuga turkestanica* have other beneficial cellular functions within aged skeletal muscle thus possibly decreasing the onset of sarcopenia (4). During sarcopenia, skeletal muscles are unable to completely repair themselves through the regeneration process (3, 6). During the skeletal muscle repair process Notch and Wnt signaling are important for satellite cell activity and regeneration (14, 65). Studies have demonstrated that C57BL/6 mice exposed to phytoecdysteroids specifically 20E, exhibited an increase in components of both Notch and Wnt signaling pathways in the triceps brachii muscle in aged mice. The increase of these pathways might help aged skeletal muscle prepare for regeneration after muscle damage (4). To be able to improve the quality of living with better muscle function it is of importance in the elderly. Although much of the research to this point demonstrates the anabolic properties of phytoecdysteroids the effects they have on different aspects of muscle regeneration are still unknown.

Role in Inflammation

Phytoecdysteroids have many effects in mammals as stated above; some may have other important effects in inflammation. Phytoecdysteroids have been shown to have potent anti-inflammatory properties when studied with pro-inflammatory factors (51, 61). If, or when, skeletal muscle becomes damaged inflammation proceeds due to one of many pro-inflammatory agents, such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (70). Sun and Yasukawa have shown that eight ecdysteroids have inhibitory effects against TPA which produced the inflammation. Another pro-inflammatory agent, carrageenan, has been shown to increase inflammation with in skeletal muscle thus decreasing muscular strength (53). Ochieng et al. (51) has shown that some phytoecdysteroids inhibit the effects of carrageenan induced inflammation on skeletal muscle. Specifically the *Ajuga* species of plants are said to have anti-inflammatory effects (39), but in what aspect is still unknown. These studies suggest that different phytoecdysteroids have anti-inflammatory properties to counteract pro-inflammatory agents within the muscle after damage.

Muscle Damage

Eccentric Contraction

One of the most common types of muscle damage is a strain or eccentric muscle damage typically caused when opposite tensile forces are present at the same time on the muscle causing damage (11). A muscle injury causes muscle soreness, movement with pain, and a limited range of motion. Many studies have used a non-invasive approach in studying muscle damage via strain or an eccentric muscle contraction model. These models are typically used to determine possible methods of treatment from a muscle injury. Such studies have examined methods such as immobilization, RICE, exercise, therapeutic ultrasound, nonsteroidal anti-inflammatory medications, and hyperbaric oxygen therapy (41).

A common model for studying eccentric muscle injury in mice is to induce injury via eccentric contractions (22, 23, 30, 68). In this model a mouse is placed on a heated platform to minimize heat loss, and then the left foot is attached to a foot pedal which is attached to a force transducer. Sterile needle electrodes are placed through the skin on either side of the common peroneal nerve, stimulation of the nerve then causes contraction of the anterior crural muscles. Electrode placement and optimal voltage are confirmed via 5-15 isometric contractions at 300 Hz with a 200-ms train duration and 0.5-ms pulses. Ultimately, injury is induced via 150 eccentric contractions. The left foot of the mouse is passively dorsiflexed to 20° and then while the muscles were stimulated, the foot was plantar flexed 40° at $2000°\cdot s^{-1}$. The anterior crural muscles are stimulated eccentrically for 20-ms trains, 0.5-ms pulses, at 300 Hz. An isometric contraction precedes the eccentric contraction which lasts 100-ms, 0.5-ms pulses, at 300 Hz. A 10-s pause is placed between each eccentric contraction and every tenth eccentric contraction torque is recorded (22, 23, 30, 68).

Inflammatory Response and Muscle Healing

After muscle injury the immune system is activated causing inflammation. There are three phases after muscle injuries. First is the destruction phase which is characterized by damage to the muscle structure and extensive damage to the vasculature within the muscle. Due to the damage of the vasculature, the inflammation response by the immune system is typically followed by cell death of the damaged muscle fibers. With an injury that only causes moderate damage, the vasculature within the muscle is typically undisrupted. The result of undisrupted vasculature allows the arterioles within the muscle to vasodilate allowing the inflammation response to commence. One result of the vasodilation allows for an increase in numbers of phagocytic leucocytes and levels of plasma proteins which are both

important for the inflammation response (62). The second phase is the repair phase, characterized by production of scar tissue and regeneration of vasculature (16). This phase works mostly through phagocytosis which will clear the necrotic tissue. Neutrophils have a very important function in providing phagocytic functions by clearing necrotic tissue as well after damage (63). They also increase the inflammation response by releasing pro-inflammatory cytokines. Although neutrophils work in clearing the necrotic tissue, they may be damaging the tissue even further (36, 37, 62). Phagocytic macrophages also help in the clearing of necrotic tissue and repairing of the muscle fibers. The same macrophages appear later in the process after the decrease in neutrophils. These macrophages trigger growth factors and cytokines to help with muscle healing (2, 49). The third and final phase is the remodeling phase, which is characterized by the formation and organization of newly formed muscle fibers as well as a decreased lesion size. This phase also constitutes an improvement in muscle function as well as angiogenesis within the muscle. Fibroblasts play a pivotal role in the regeneration of muscle tissue by secreting extracellular proteins such as growth factors (59, 60).

In a muscle healing study by Khattack et al. (42) after an injury, it was observed that in the mobilization (allowed to move freely) group there was an increase in myotubel formation as well as a decrease in the inflammation response that may result in a better healing response (42). These data suggests that if the inflammation response is decreased by mobilizing the limb, then the muscle will heal quicker than if it was immobilized (placed in a cast). Other studies examined muscle healing through supplementation of suramin two weeks after contusion and strain injuries (19, 50). Suramin is an antiparasitic and antineoplastic agent which has been shown to enhance muscle regeneration after strain and laceration

injuries (18, 19). Two weeks after muscle contusion with the supplementation of suramin showed an increase in regenerative muscle fibers. Suramin injection can also increase strength and muscle function after a muscle injury (19). Chan et. al (19), also showed an increased number of regenerating myofibers compared to control groups after strain injury. Furthermore, suramin also recovered fast-twitch strength to control values with injection of 5.0 mg suramin (19). A study which examined the effects of a nonsteroidal anti-inflammatory drug (NSAID), flurbiprofen, showed an increases in maximal torque recovery at three and seven days but a decline in torque at 28 days when treated with the NSAID (27). These authors suggest that with flurbiprofen administration the inflammation process is delayed and ultimately causes secondary muscle damage which would be an explanation for the late decline in torque (27). Another study exhibited that supplementation of proanthocyanidolic oligomers (PCO) blunted the neutrophil infiltration response while allowing earlier macrophage infiltration into the tissue (43). Kruger and Smith (43) saw quickened muscle regeneration due to earlier recruitment of activated satellite cells and modulation of the immune system for anti-inflammatory status when supplementing short term with PCO. This may provide a natural alternative for anti-inflammatory treatments but more research is warranted on the cellular mechanisms in how PCO affects muscle healing. These studies suggest that an intervention with medication or natural agents can increase the healing process and recover force-generating capabilities.

Contractile Function: In Vivo Contractile Function After Injury

After a muscle injury the muscle may not be able to move without pain, limited range of motion, or just muscle soreness (60). After an eccentric muscle contraction induced injury, the immediate post-injury *in vivo* maximal tetanic torque production has been reported to

decrease by 55% compared to the pre-injury value (68). Furthermore, at 3 days post-injury, maximal tetanic torque was attenuated by about 50% compared to pre-injury. At day 14, maximal tetanic torque was attenuated by 10% and maximal tetanic torque was fully recovered 21 days after injury (68). Another study measured a 50% decrease of maximal tetanic torque production immediately after injury (23). A decrease of about 53% has also been reported immediately post-injury in maximal tetanic torque production, and the values were still attenuated at 14 days post-injury (22). These studies suggest there is an immediate and prolonged decrease in maximal tetanic torque production after eccentric contraction induced skeletal muscle damage to the anterior crural muscles.

Protein Synthesis After a Mechanical Stimulus

Recent research has shown a mechanical stimulus of skeletal muscle such as overload, concentric or eccentric contractions increase protein synthesis via an activation of mTOR and other downstream intracellular signaling mediators (13, 71). Firstly, mTOR is activated by Akt via phosphorylation of tuberous sclerosis complex-2 (Tsc2) thus activating mTOR (38). A more recent study has suggested that after eccentric contractions mTOR is stimulated via Tsc2 (40). The mTOR pathway has also been reported to be activated after high frequency stimulation of rat muscles (13). Activation of mTOR in turn stimulates p70s6 kinase (p70s6k) (15). In high frequency lengthening contractions p70s6k is phosphorylated (5). This event ultimately leads to inhibition of eukaryotic initiation factor 4 binding protein (4E-BP1) which is a negative regulator of protein synthesis (34, 54, 71). The phosphorylation of both p70s6k and 4E-BP1 lead to an increased rate of translation initiation for protein synthesis (44). It is thought that p70s6k leads to phosphorylation of ribosomal protein S6 (rpS6) as well, which leads to an increase in protein synthesis after resistance exercise (17,

66). Phosphorylation of rpS6 has been used as an indirect indicator of mTOR activation but the exact role rpS6 plays in regulation of protein synthesis is still to be determined fully (29).

Studies have investigated the effects of eccentric contraction induced injuries on protein synthesis rates after injury. Lowe et al. (48) reported that after eccentric muscle damage protein synthesis rates begin to increase 2 days post injury (48). At three and five days post-injury protein synthesis rates were increased by about 30% and 40% respectively (48). Furthermore, after high frequency eccentric contractions between 12-17 hours protein synthesis rates of the TA was increased 41% (69). Wong and Booth (69) also measured a 52-65% increase in protein synthesis at 36-41 hours post eccentric exercise. These studies suggest that increased protein synthesis is required for muscle regeneration as well as a recovery of torque and force production after injury (9, 48, 57, 69).

Anabolic Agent Effects on Healing

Anabolic agents such as testosterone cause an increase in muscle mass and strength when supplemented in humans. When it comes to the effects of anabolic agents on healing after a muscle injury very little research has been conducted. One of the few studies with anabolic steroids and corticosteroids observed several important factors (10). First, anabolic steroids did not increase muscle healing in respect to twitch and tetanic force production acutely (10). However, there was a trend for chronic anabolic steroid treatment to increase twitch and tetanic force production. Corticosteroids acutely reduce the inflammation response by decreasing the amount of inflammatory cells (10). Acute corticosteroid treatment did increase acute twitch and tetanic force when compared to the anabolic steroid and control groups (10). Chronically, corticosteroid treatment does not improve healing from injury (10). This study observed that corticosteroids caused atrophy within the muscle as the time frame

was prolonged (10). More research is warranted on the effects of different doses of corticosteroids to determine how atrophy is affecting the healing process (10). However, unlike typical anabolic steroids, 20E has been reported to utilize a non-androgenic receptor and still increase growth during the regeneration process (64).

Many studies have utilized the eccentric contraction model to simulate injury for study of the muscle healing process. Muscle injury results in the loss of strength, functional movement and possibly muscle mass. Phytoecdysteroids have been shown to increase protein synthesis and have possible anabolic properties. Very few studies have examined the effects of anabolic agents on muscle damage that was induced by an eccentric muscle contraction. This warrants further research to determine the possible healing effects of phytoecdysteroids on an eccentric muscle contraction induced injury.

Chapter 3: Methods

Animals

Male C57BL/6 mice (n = 32) 3-to 6-months of age were randomly assigned to one of four groups (n = 8/group), see Figure 1. The mice in all groups underwent an eccentric muscle contraction injury protocol and the muscle repair process was investigated with or without phytoecdysteroid supplementation. The mice were kept on a normal 12h: 12h light-dark cycle, food, and water were provided ad libitum.

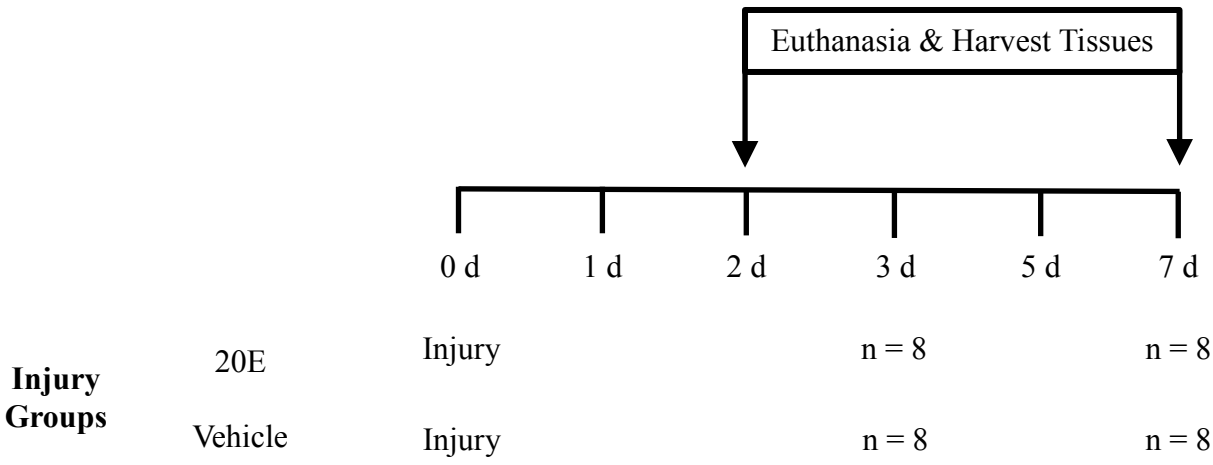

Figure 1. Protocol schematic for experimental design. **Injury* – indicates when mice were injured.

Experimental Design

Mice underwent the eccentric muscle contraction injury protocol to the anterior crural muscles after which they were supplemented with 50mg·kg^{-1} body weight (BW) (32, 47) of 20E or placebo up to 7 days. The day of injury, an *in vivo* torque-frequency protocol was utilized before and after injury to assess the submaximal to maximal torque response. The

injury was induced via 150 eccentric skeletal muscle contractions. The day of euthanasia, the torque-frequency protocol was administrated. Following contractile function measurements, animals were euthanized and skeletal muscle was dissected, weighed, and stored at -80° C until further analysis of intracellular signaling and protein synthesis via Western blot analysis. All experiments and procedures were approved (12/12/2016) by the Appalachian State University Institutional Animal Care and Use Committee (#17-09).

In Vivo Torque-Frequency and Eccentric Muscle Damage

A commonly used model of *in vivo* contractile function and eccentric muscle damage was utilized for this experiment, as previously described by Corona et al. (22). Mice were weighed prior to being anesthetized via inhalation of 4% isoflurane with 800 mL/min oxygen (O_2) and maintained at 2% inhaled isoflurane with 500 mL/min O_2 output throughout the duration of the experiment. Once anesthetized the hair on both hind limbs was removed with a depilation cream, thus allowing the anterior crural muscles to be better visualized through the skin. The mice were then placed on a heated platform to maintain body temperature and the left foot was secured to the foot pedal connected to the computer controlled servomotor (Model 305B-LR; Aurora Scientific, Richmond Hill, Ontario, Canada). Sterilized electrode needles were placed percutaneous for stimulation of the left common peroneal nerve. Optimal electrode placement was determined using 5-15 isometric contractions (200-ms train, 0.1-ms pulses at 300 Hz). An *in vivo* torque-frequency protocol was utilized to assess contractile function of the anterior crural muscles at increasing frequencies (1-300 Hz) with 2 minutes rest in between contractions before injury, 5 minutes after injury, and either 3 or 7 days post-injury. Injury to the anterior crural muscles was induced via 150 eccentric muscle contractions (300 Hz, 120-ms train of 0.1-ms pulses with 38° angular movement at

2000°·sec^{-1}). Peak eccentric torque was measured at every tenth eccentric contraction. The right leg was then secured to the foot pedal, optimal electrode placement was found, and the eccentric muscle damage protocol was administered. The mice were then returned to individual cages and allowed to recover. At either 3 or 7 days post-injury, the mice were anesthetized as described above and an *in vivo* torque-frequency protocol was administered to the left hind limb to assess contractile function of the anterior crural muscles. After contractile function assessment the mice were injected interperitionel with puromycin (0.040 µmol·g^{-1} BM) 30 minutes prior to euthanasia for assessment of protein synthesis via Western blot analysis. Mice were euthanized via cervical dislocation while under anesthesia and confirmed dead via removal of the heart before dissection of skeletal muscles. The anterior crural skeletal muscles (tibialis anterior and extensor digatorum longus) were harvested from both hind limbs and wet weights were measured. The left hind limb skeletal muscles were either frozen in liquid nitrogen (N_2) cooled isopentane and the right hind limb skeletal muscles were snap frozen in N_2 and stored in a -80°C freezer until further analysis.

Phytoecdysteroid Supplementation

After receiving the eccentric muscle injury and after recovery from the anesthetic on day zero, the mice were supplemented phytoecdysteroid 20-hydroxyecdysone (20E) (50mg·kg^{-1} BW or 0 mg·kg^{-1} BW); Bosche Scientific, (E6425-HE; New Brunswick, NJ, USA). The dose of 20E or placebo was delivered via a liquid diet supplement (Liquid Diet; cat #F1268 BioServ, Flemington, NJ). The liquid diet is a nutritionally complete diet that contains 17.5% protein; 5.4% fat; 4.8% fiber; and 67.7% carbohydrate. The previous day's BW was used to calculate the appropriate dosage of 20E mixed with the liquid diet supplement. The required amount of 20E was dissolved in warm tap water and placed into a

single serving of the liquid diet (placebo). The liquid diet with or without 20E was served to the mice via gavage which was calculated as 1% of the BW of the mice.

Western Blot Analysis

Right tibialis anterior and extensor digitorum longus skeletal muscles were homogenized, as previously done in our lab (47), on ice in 0.3 ml homogenization buffer containing 150.0 mM NaCl, 1.0% Triton-X, 0.5% sodium deoxycholate, 10 mM Tris-HCl, pH 7.4 with protease and phosphatase inhibitor cocktails (Sigma Aldrich; St. Louis, MO, USA). After homogenization, samples were cleared by centrifugation of the homogenate at 14,000 *x g* for 5 minutes @ 4°C. The supernatant was removed and protein concentration was assessed via BCA protein assay (Pierce; Thermo Fisher Scientific, Inc.; Rockford, IL, USA). Total protein (30 µg) was prepared using a Laemmli buffer and subjected to electrophoresis separation by SDS-PAGE on 4-15% TGX-stain free acrylamide gels (Catalog # 5678084; Bio-Rad; Hercules, CA, USA) as previously described (35). After completion of the electrophoresis the proteins were transferred to a polyviylidene difluoride (PVDF) membrane (Millipore; Bradford, MA, USA) and imaged under ultraviolet light to ensure equal loading and transferring of proteins, as well as to quantitate total protein for normalization. Membranes were blocked using 5% nonfat dry milk in wash buffer [1X Tris-buffered saline (TBS) with 0.1% Tween20] for one hour at room temperature. Following blocking, the membranes were washed two times and immediately incubated sequentially with the following primary antibodies overnight @ 4°C: anti-puromycin [MillporeSigma; Billerica, MA, USA – MABE343, clone 12D10; 1:5000 in 1% bovine serum albumin (BSA)], as well as phosphorylated Akt^{Ser473} (Cell Signaling; Danver, MA, USA – 9271); phosphorylated 4E-BP1$^{Thr37/46}$ (Cell Signaling – 2855); and phosphorylated rpS6$^{Ser235/236}$

(Cell Signaling – 2211), (all signaling antibodies = 1:1000 in 5% BSA). After primary antibody incubation, membranes were washed 5 times for 5 minutes each in wash buffer and then incubated with secondary antibodies for 1 hour at room temperature (1:50,000 for puromycin and 1:20,000 for signaling antibodies). Next, the membranes were washed 5 times for 5 minutes each in wash buffer. Membranes were exposed to SuperSignal West Dura ECL chemiluinescent detection HRP reagents (Pierce; Thermo Fisher Scientific, Inc.; Rockford, IL, USA) mixed 1:1 for 5 minutes. Membranes were then imaged and densitometry analyzed using the ChemiDoc XRS+ molecular imaging system and analysis software (BioRad).

Data Acquisition and Analysis

The muscle lever system, stimulator and force transducer was connected to a signal interface (Model 610A, Aurora Scientific, Aurora, ON, Canada) that sent the analog signal to an analog to digital converter card (Model PCI-6221, National Instruments, Austin, TX, USA) on a computer with Dynamic Muscle Control software (Aurora Scientific, Aurora, ON, Canada). The force output data was analyzed utilizing the Dynamic Muscle Analysis software (Aurora Scientific, Aurora, ON, Canada).

Statistics

All values are expressed as mean ± SE, and statistical significance was set at $p < 0.05$. A Bonferroni correction was to determine statistical significance and was set at $p < 0.00625$. A univariate test was used to determine differences in protein synthesis rates, phosphorylation of: Akt, 4E-BP1, and rpS6, between the 20E and the placebo group. A three-way ANOVA with repeated measures was used to evaluate the recovery of muscle torque-generating capacity. Treatment group, harvest time post-injury, and stimulation frequency

constituted the three factors. A three-way interaction was assessed for differences within groups (group x harvest day x time). This was utilized for determining differences in the recovery of torque generating capacity of the muscles. A least significant difference *post-hoc* analysis was used to determine differences within/between groups. Data was analyzed using SPSS 24.0 for windows (SPSS Inc., Chicago, IL, USA).

Chapter 4: Results

In Vivo Contractile Function

Isometric twitch (1 Hz) torque production is a single submaximal stimulation and is reported as (mN/kg BW). The pre-isometric twitch torque was compared to the post-isometric twitch torque, this was also done at other specific frequencies as well (i.e., 20, 40, 200 Hz). Comparing the twitch isometric torque of the 3-day placebo group before injury to post-injury there was a significant difference in torque production (Table 1). All the other groups at the other frequencies were significantly different from pre-to post-injury, with the pre-injury being significantly greater than post (Table 1).

Twitch torque is the same between both 3-day placebo and 20E groups before induction of eccentric muscle injury (Table 2). After the eccentric muscle injury protocol, the isometric twitch torque production was the same for placebo and 20E groups immediately post-injury (Table 2). At 3 days post-injury there was not a difference in torque generating capacity (Table 2). Likewise, with the 7-day placebo and 20E groups, before injury, immediately post-injury, and 7 days post-injury; there was not difference in torque generating capacity within groups (Table 2). At the remaining frequencies, 20 Hz, and 40Hz, no differences are apparent within those groups respectively (Table 2). At 200 Hz pre-injury, immediately post-injury, and the 3-day placebo and 20E groups are not different (Table 2). The same occurred within the 7-day placebo and 20E groups (Table 2).

Table 1. Contractile Function Pre to Post-Injury

Variable	Pre-Injury	Immediately Post-Injury	Time; Interaction P values
Torque			
Torque 1 Hz - 3 Day (mN/kg)			
Placebo	19.96 ± 5.44	7.650 ± 1.18	0.001 ; 0.249
20E	17.41 ± 5.82	4.986 ± 1.26	
Torque 1 Hz - 7 Day (mN/kg)			
Placebo	22.70 ± 5.44	8.400 ± 1.18	0.001 ; 0.249
20E	11.54 ± 5.44	7.013 ± 1.18	
Torque 20 Hz - 3 Day (mN/kg)			
Placebo	25.35 ± 3.45	8.838 ± .977	0.000 ; 0.851
20E	22.21 ± 3.69	5.929 ± 1.04	
Torque 20 Hz - 7 Day (mN/kg)			
Placebo	28.16 ± 3.46	9.363 ± .977	0.000 ; 0.851
20E	28.02 ± 3.46	8.075 ± .977	
Torque 40 Hz - 3 Day (mN/kg)			
Placebo	27.00 ± 3.68	9.775 ± 1.01	0.000 ; 0.843
20E	23.32 ± 3.94	6.114 ± 1.08	
Torque 40 Hz - 7 Day (mN/kg)			
Placebo	31.11 ± 3.68	10.84 ± 1.01	0.000 ; 0.843
20E	30.22 ± 3.68	8.475 ± 1.01	
Torque 200 Hz - 3 Day (mN/kg)			
Placebo	104.5 ± 8.79	76.90 ± 6.86	0.000 ; 0.207
20E	101.9 ± 9.39	64.39 ± 7.33	
Torque 200 Hz - 7 Day (mN/kg)			
Placebo	128.2 ± 8.79	65.41 ± 6.86	0.000 ; 0.207
20E	124.8 ± 8.79	72.78 ± 6.86	

Data are means ± SEM. 20E = 20-Hydroxyecdysone.

Table 2. Contractile Function

Variable	Pre-Injury	Immediately Post-Injury	Harvest Day	Time; Interaction P values
Torque				
Torque 1 Hz - 3 Day (mN/kg)				
Placebo	19.96 ± 5.44	7.650 ± 1.18	14.53 ± 3.27	0.001 ; 0.317
20E	17.41 ± 5.82	4.986 ± 1.26	6.886 ± 3.49	
Torque 1 Hz - 7 Day (mN/kg)				
Placebo	22.70 ± 5.44	8.400 ± 1.18	18.55 ± 3.27	0.001 ; 0.317
20E	11.54 ± 5.44	7.013 ± 1.18	12.88 ± 3.27	
Torque 20 Hz - 3 Day (mN/kg)				
Placebo	25.35 ± 3.45	8.838 ± .977	14.78 ± 2.89	0.000 ; 0.910
20E	22.21 ± 3.69	5.929 ± 1.04	11.20 ± 3.09	
Torque 20 Hz - 7 Day (mN/kg)				
Placebo	28.16 ± 3.46	9.363 ± .977	20.66 ± 2.89	0.000 ; 0.910
20E	28.02 ± 3.46	8.075 ± .977	16.86 ± 2.89	
Torque 40 Hz - 3 Day (mN/kg)				
Placebo	27.00 ± 3.68	9.775 ± 1.01	15.31 ± 2.96	0.000 ; 0.960
20E	23.32 ± 3.94	6.114 ± 1.08	11.34 ± 3.17	
Torque 40 Hz - 7 Day (mN/kg)				
Placebo	31.11 ± 3.68	10.84 ± 1.01	22.13 ± 2.96	0.000 ; 0.960
20E	30.22 ± 3.68	8.475 ± 1.01	18.94 ± 2.96	
Torque 200 Hz - 3 Day (mN/kg)				
Placebo	104.5 ± 8.79	76.90 ± 6.86	97.58 ± 10.6	0.000 ; 0.022
20E	101.9 ± 9.39	64.39 ± 7.33	72.71 ± 11.3	
Torque 200 Hz - 7 Day (mN/kg)				
Placebo	128.2 ± 8.79	65.41 ± 6.86	97.40 ± 10.6	0.000 ; 0.022
20E	124.8 ± 8.79	72.78 ± 6.86	117.3 ± 10.6	

Data are means ± SEM. 20E = 20-Hydroxyecdysone.

Protein Synthesis and Signaling

Markers of protein synthesis and intracellular signaling activity (Akt, 4E-BP1, rpS6) were assessed via Western blot analysis and are expressed as arbitrary units (AU). The total phosphorylation status of Akt^{Ser473}, 4E-BP1$^{Thr37/46}$, rpS6$^{Ser235/236}$ was assessed in the right leg (non-contractile leg) for both the tibialis anterior (TA) and extensor digitorum longus (EDL) skeletal muscles in groups of n = 8 except for the 3 day 20E group, which had an n = 7.

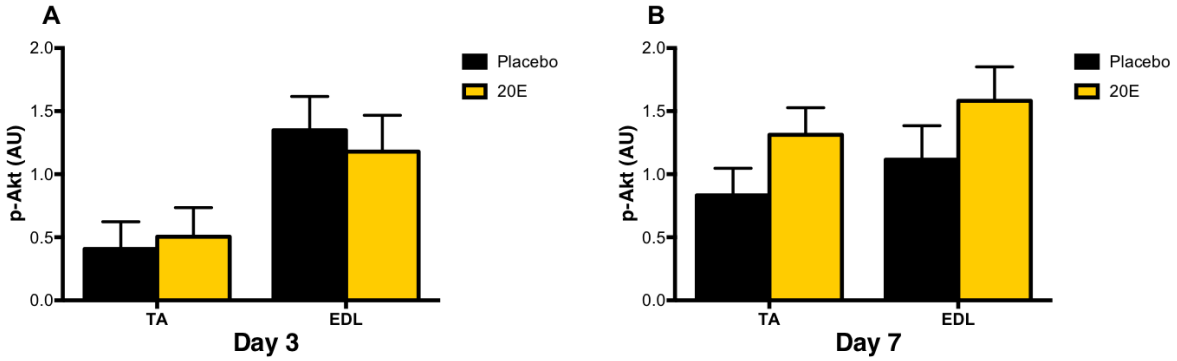

Figure 2. Phosphorylated Akt^{Ser473} for the right leg at 3 days post-injury (A) and 7 days post-injury (B).

No significant difference was observed in phosphorylation of Akt^{Ser473} in the TA 3 days post-injury (p = 0.759) or 7 days post-injury (p = 0.128) and EDL 3 days post-injury (p = 0.673)

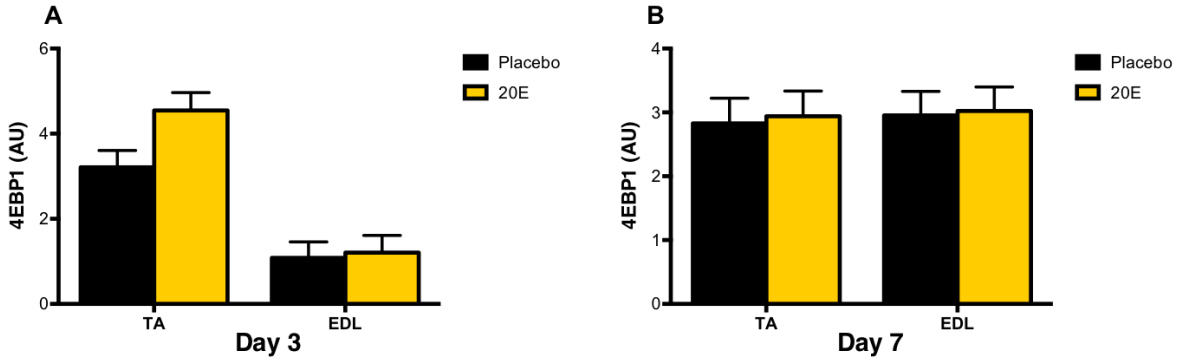

Figure 3. Phosphorylated 4E-BP1$^{Thr37/46}$ for the right leg at 3 days post-injury (A) and 7 days post-injury (B).

or 7 days post-injury (p = 0.230) (Figure 2A and 2B). In addition, no significant differences are observed in phosphorylation of 4E-BP1$^{Thr37/46}$ for the TA 3 days post-injury (p = 0.029) or 7 days post-injury (p = 0.842) (Figure 3A and 3B). Furthermore, no significant difference was observed in phosphorylation of 4E-BP1$^{Thr37/46}$ for the EDL 3 days post-injury (p = 0.821) or 7 days post-injury (p = 0.897) (Figure 3A and 3B). No differences are observed in

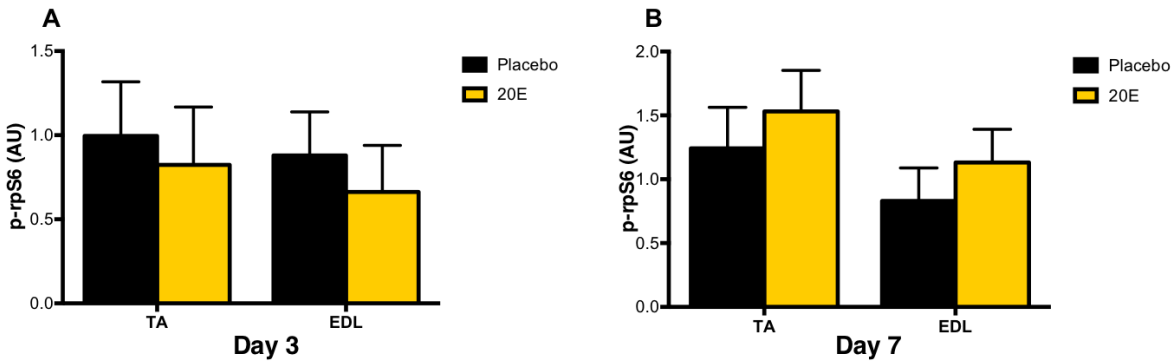

Figure 4. Phosphorylated rpS6$^{Ser235/236}$ for the right leg at 3 days post-injury (A) and 7 days post-injury (B).

phosphorylation of rpS6$^{Ser235/236}$ in the TA at 3 days post-injury (p = 0.716) or 7 days post-injury (p = 0.529) or in the EDL 3 days post-injury (p = 0.574) or 7 days post-injury (p = 0.415) (Figures 4A and 4B). Lastly, no significant differences were observed in total protein synthesis (puromycin) in the TA 3 days post-injury (p = 0.701) or 7 days post-injury (p = 0.190), as well as in the EDL 3 days post-injury (p = 0.797) and 7 days post-injury (p = 0.931) (Figures 5A and 5B). Post-injury (p = 0.701) or 7 days post-injury (p = 0.190), as well as in the EDL 3 days post-injury (p = 0.797) and 7 days post-injury (p = 0.931) (Figures 5A and 5B).

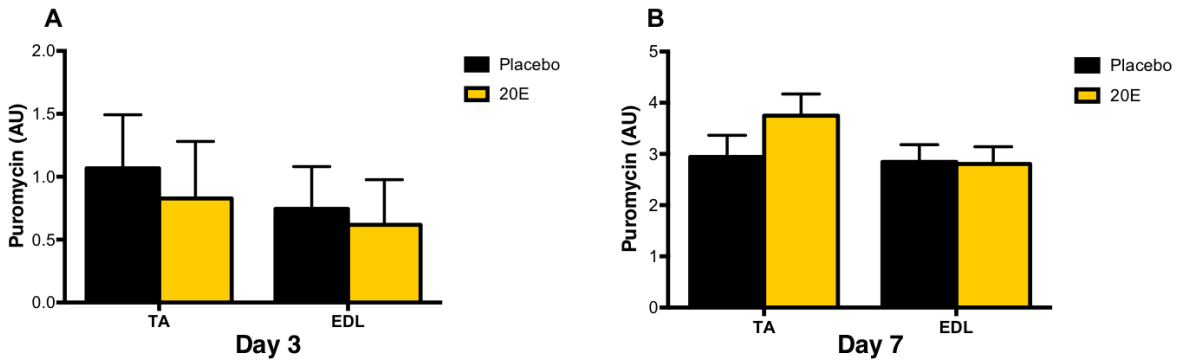

Figure 5. Total protein synthesis (puromycin) for the right leg at 3 days post-injury (A) and 7 days post-injury (B).

Chapter 5: Discussion

The purpose of the current study was to determine the degree to which the phytoecdysteroid 20E, (1) enhances recovery of maximal tetanic torque production and (2) additively increases protein synthesis after a bout of eccentric contraction muscle injury. This study sought to test the hypothesis that 20E would (1) accelerate the rate of recovery of muscle maximal tetanic torque production and (2) additively increase the rate of protein synthesis after an eccentric contraction muscle injury to the anterior crural muscles compared to injured mice not receiving 20E treatment.

In Vivo Injury Induction

At all frequencies (1, 20, 40, 200 Hz) pre-to post-injury there was a significant decrease in torque production by the anterior crural muscles. This is consistent with other studies that have measured a significant decrease in isometric torque as a function of stimulation frequency (22). Corona et al. (22) reported a 52.7% - 92.1% decrease in isometric torque after eccentric muscle injury. We report decreases torque production in the 3-day placebo group immediately post-injury at 1 Hz, 20 Hz, 40 Hz, and 200 Hz of ~62%, ~65%, ~63% and ~26% respectively (Figure 6A). Furthermore, decreases in torque production were observed in the 3-day 20E group immediately post-injury at 1 Hz, 20 Hz, 40 Hz, and 200 Hz of ~72%, ~73%, ~74% and ~37% respectively (Figure 6B). Recovery of torque production was still decreased at 3-days post-injury in the placebo group, at the same frequencies, ~27%, ~42%, ~43%, and ~6% respectively (Figure 6A). Lastly, torque production was still decreased at 3-days post-injury in the 20E group at 1 Hz, 20 Hz, 40 Hz, and 200 Hz of ~60%, ~50%, ~51% and ~29% respectively (Figure 6B). In contrast Corona et al. (22) reported an immediately post-injury torque production decrease of ~82%, ~83%, ~81% and ~61% at 1,

20, 40 and 200 Hz respectively. At 3-days post-injury, at the same frequencies, Corona et al. (22) reported decreases in torque productions of ~82%, ~73%, ~79% and ~70% compared to pre-injury values. Our lab did not see the same immediate decrease in toque production in the 3-day group and our 3-day torque production was still greater than that of what Corona et al. (22) reported. In our 7-day placebo group a decrease in torque production was observed immediately post-injury by ~63%, ~67%, ~65% and ~49% at 1, 20, 40 and 200 Hz respectively (Figure 6A). In the 7-day 20E group, torque production was decreased

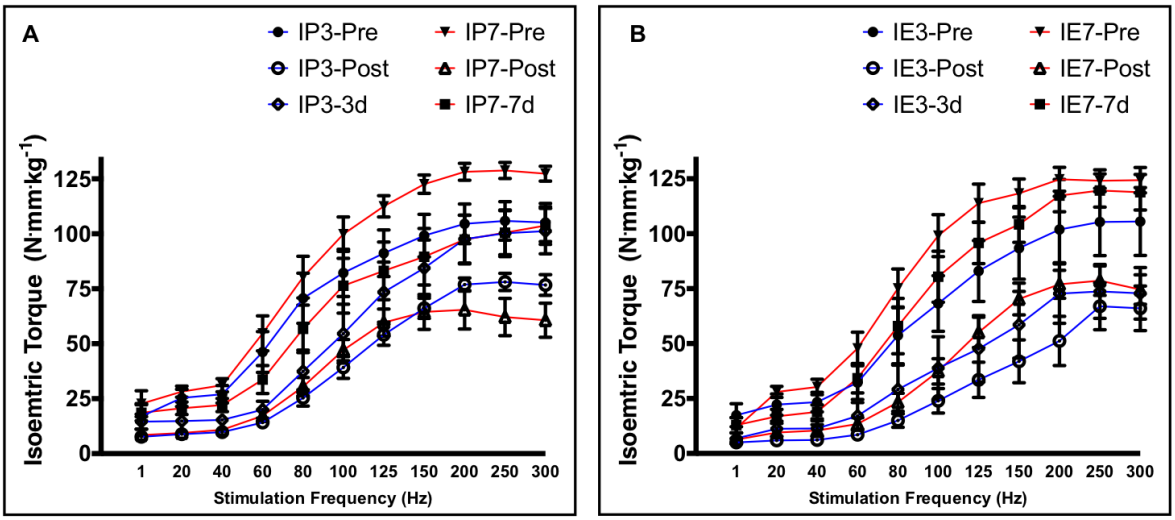

Figure 6. Isometric torque frequency curve for placebo groups (A) and isometric torque frequency curve for 20E groups (B).

immediately post-injury by ~40%, ~71%, ~72% and ~42% at 1, 20, 40 and 200 Hz respectively (Figure 6B). Torque production was still decreased compared to pre-injury values at 7-days post-injury in the placebo group by ~18%, ~27%, ~29% and ~24% at 1, 20, 40 and 200 Hz respectively (Figure 6A). Lastly, in the 7-day 20E group, torque production

was increased compared to pre-injury values at 1 Hz by ~12% (Figure 6B). At the frequencies 20, 40 and 200 Hz the torque production was still decreased by ~40%, ~37% and ~6% respectively (Figure 6B). Corona et al. (22) reported a reduction in torque production at 7-days post-injury of ~64%, ~60%, ~52% and ~20% at 1, 20, 40 and 200 Hz respectively. These comparisons suggest that we saw a slightly more rapid recovery of torque production at different time points compared to the data of Corona et al. (22). Warren et al. (67) also reported a decrease of about 55% pre to post-injury torque production after eccentric contractions. This decrease in isometric torque production is indicative of anterior crural muscle injury and not fatigue (67). This study supports the notion that the injury protocol induced skeletal muscle injury and not fatigue because of eccentric contractions.

***In Vivo* Protein Synthesis, Signaling, and Contractile Function**

Phytoecdysteroids and their anabolic effects have been well documented (1, 7, 20, 25, 31, 32, 39, 47, 56, 64). Yet there is still some conflicting results between 20E and increases in muscle protein synthesis (20, 47, 64). Some of the conflicting results are differences in dosage, administration, and length of treatment. However, the current study is the first to use this potentially anabolic agent after an eccentric contraction protocol to assess torque production recovery and protein synthesis.

To determine if 20E additively increases protein synthesis targets that are thought to be phosphorylated after eccentric contractions as well as with the supplementation of phytoecdysteroids were assessed. These targets were Akt^{Ser473}, $4E\text{-}BP1^{Thr37/46}$, and $rpS6^{Ser235/236}$ as well as overall protein synthesis rates via puromycin. To test for activation of these signaling molecules the right TA and EDL that were only injured and did not undergo a torque-frequency immediately prior to tissue harvest were utilized. We did not detect any

differences in activation of either Akt^{Ser473}, 4E-BP1$^{Thr37/46}$, or rpS6$^{Ser235/236}$ between treatments. 20E has shown to activate the PI3k-Akt pathway (31, 32), but downstream mediators of Akt and mTOR such as 4E-BP1 and rpS6 have not all been investigated with 20E supplementation alone (1). After eccentric contractions protein synthesis rates have been shown to be increased at 3 days post-injury by 30% and at 5 days post-injury by 40% (48). The reason for no additional increase in phosphorylation of Akt^{Ser473}, 4E-BP1$^{Thr37/46}$, or rpS6$^{Ser235/236}$ could be due to having missed the peak of acute signaling since the mice were not supplemented the day of tissue harvest. 20E has an eight minute half-life and can be eliminated in about 30 minutes (28), which may explain the reason for the missed signaling. The current study set out to investigate the chronic signaling effects (days) and not the acute signaling effects (hours). Another possible reason could be due to a low dosage amount.

We utilized a dosage of 50mg/kg BW of 20E, which has been shown to increase protein synthesis, as well as grip strength in rats (32). Anthony et al. (1) suggested that dosages of 10, 50, and 200 mg/kg of 20E do not significantly alter the phosphorylation status of Akt, mTOR or 4E-BP1 with just gavaging alone. A certain type of stimuli may be needed to alter the phosphorylation status of these proteins. Again, in the current study, mice were gavaged with 50 mg/kg of 20E, but with the increase in protein synthesis due to the eccentric contractions (9, 48) an increased dosage may be warranted to additively increase protein synthesis. A limitation of the current study is that no true control non-injured mice were used to compare protein synthesis rates. Without the non-injured control mice we are unable to determine if the eccentric contractions induced an increase in overall protein synthesis without relation to the treatments.

We also investigated the torque production capabilities with or without 20E supplementation after eccentric muscle injury. When examining the different specific frequencies (1, 20, 40, 200 Hz) there was not a consistent significant difference within groups. Torque generating capabilities were the same between the placebo and 20E groups at 3 days post-injury, the same held true for 7 days post-injury. In addition, there were minimal differences when investigating other frequencies such as 20, 40, and 200 Hz. The lack of consistent differences could be attributed to not having an increased protein synthesis in the 20E group compared to the placebo group. It has been reported that an increase in mTOR and p70s6k1 phosphorylation increases after eccentric contractions and mTOR is important for recovery of peak torque (9). Anthony et al. (1) indicated that mTOR was not phosphorylated with 20E alone, which could indicate why we did not see any phosphorylation resulting from the 20E supplementation.

Conclusions

This study is the first to investigate the effects of 20E on torque production, protein synthesis rates and intracellular signaling after eccentric muscle injury. Contrary to my hypothesis, 20E does not additively increase protein synthesis or accelerate recovery of muscle function after eccentric muscle injury. However, further research is needed to determine the appropriate dosage to elicit an additive effect after eccentric muscle injury.

References

1. **Anthony TG, Mirek ET, Bargoud AR, Phillipson-Weiner L, DeOliveira CM, Wetstein B, Graf BL, Kuhn PE, and Raskin I**. Evaluating the effect of 20-hydroxyecdysone (20HE) on mechanistic target of rapamycin complex 1 (mTORC1) signaling in the skeletal muscle and liver of rats. *Appl Physiol Nutr Metab* 40: 1324-1328, 2015.
2. **Arnold L, Henry A, Poron F, Baba-Amer Y, Van Rooijen N, Plonquet A, Gherardi R, and Chazaud B**. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *Journal of Experimental Medicine* 204: 1057-1069, 2007.
3. **Arthur ST, and Cooley ID**. The effect of physiological stimuli on sarcopenia; impact of Notch and Wnt signaling on impaired aged skeletal muscle repair. *International journal of biological sciences* 8: 731-760, 2012.
4. **Arthur ST, Lila MA, Keith MD, Zwetsloot KA, Grace MH, Demick JL, Davenport ME, Lawrence MM, Howden R, Badmaev V, Blanton SE, and Shanely RA**. Ajuga turkestanica increases Notch andWnt signaling in aged skeletal muscle. *European review for medical and pharmacological sciences* 18: 9, 2014.
5. **Baar K, and Esser K**. Phosphorylation of p70S6k correlates with increased skeletal muscle mass following resistance exercise. *American Journal of Physiology - Cell Physiology* 276: C120-C127, 1999.
6. **Ballak SB, Degens H, de Haan A, and Jaspers RT**. Aging related changes in determinants of muscle force generating capacity: a comparison of muscle aging in men and male rodents. *Ageing Res Rev* 14: 43-55, 2014.
7. **Bathori M, Toth N, Hunyadi A, Marki A, and Zador E**. Phytoecdysteroids and anabolic-androgenic steroids--structure and effects on humans. *Curr Med Chem* 15: 75-91, 2008.
8. **Báthory M, Tóth I, Szendrei K, and Reisch J**. Ecdysteroids in Spinacia oleracea and Chenopodium bonus-henricus. *Phytochemistry* 21: 236-238, 1982.
9. **Baumann CW, Rogers RG, Otis JS, and Ingalls CP**. Recovery of strength is dependent on mTORC1 signaling after eccentric muscle injury. *Muscle Nerve* 54: 914-924, 2016.
10. **Beiner JM, Jokl P, Cholewicki J, and Panjabi MM**. The effect of anabolic steroids and corticosteroids on healing of muscle contusion injury. *Am J Sports Med* 27: 2-9, 1999.
11. **Beltran L, Ghazikhanian V, Padron M, and Beltran J**. The proximal hamstring muscle-tendon-bone unit: a review of the normal anatomy, biomechanics, and pathophysiology. *Eur J Radiol* 81: 3772-3779, 2012.
12. **Bergamasco R, and Horn D**. Distribution and role of insect hormones in plants. *Endocrinology of insects* 1: 627-654, 1983.
13. **Bolster DR, Kubica N, Crozier SJ, Williamson DL, Farrell PA, Kimball SR, and Jefferson LS**. Immediate response of mammalian target of rapamycin (mTOR)-mediated signalling following acute resistance exercise in rat skeletal muscle. *J Physiol* 553: 213-220, 2003.
14. **Brack AS, Conboy IM, Conboy MJ, Shen J, and Rando TA**. A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell stem cell* 2: 50-59, 2008.
15. **Brunn GJ, Fadden P, Haystead TAJ, and Lawrence JC**. The Mammalian Target of Rapamycin Phosphorylates Sites Having a (Ser/Thr)-Pro Motif and Is Activated by Antibodies to a Region near Its COOH Terminus. *Journal of Biological Chemistry* 272: 32547-32550, 1997.
16. **Bunn JR, Canning J, Burke G, Mushipe M, Marsh DR, and Li G**. Production of consistent crush lesions in murine quadriceps muscle—A biomechanical, histomorphological and immunohistochemical study. *Journal of Orthopaedic Research* 22: 1336-1344, 2004.

17. **Burd NA, Holwerda AM, Selby KC, West DWD, Staples AW, Cain NE, Cashaback JGA, Potvin JR, Baker SK, and Phillips SM**. Resistance exercise volume affects myofibrillar protein synthesis and anabolic signalling molecule phosphorylation in young men. *The Journal of Physiology* 588: 3119-3130, 2010.
18. **Chan Y-S, Li Y, Foster W, Horaguchi T, Somogyi G, Fu FH, and Huard J**. Antifibrotic effects of suramin in injured skeletal muscle after laceration. *Journal of Applied Physiology* 95: 10, 2003.
19. **Chan YS**. The Use of Suramin, an Antifibrotic Agent, to Improve Muscle Recovery After Strain Injury. *American Journal of Sports Medicine* 33: 43-51, 2005.
20. **Cheng DM, Kutzler LW, Boler DD, Drnevich J, Killefer J, and Lila MA**. Continuous infusion of 20-hydroxyecdysone increased mass of triceps brachii in C57BL/6 mice. *Phytotherapy research : PTR* 27: 107-111, 2013.
21. **Cheng DM, Yousef GG, Grace MH, Rogers RB, Gorelick-Feldman J, Raskin I, and Lila MA**. In vitro production of metabolism-enhancing phytoecdysteroids from Ajuga turkestanica. *Plant Cell Tiss Org* 93: 73-83, 2008.
22. **Corona BT, Rouviere C, Hamilton SL, and Ingalls CP**. FKBP12 deficiency reduces strength deficits after eccentric contraction-induced muscle injury. *J Appl Physiol (1985)* 105: 527-537, 2008.
23. **Dawn A. Lowe GLW, Christopher P. Ingalls, Daniel B. Boorstein, R. B. Armstrong**. Muscle function and protein metabolism after initiation of eccentric contraction-induced injury. *Journal of Applied Physiology* 79: 1260-1270, 1995.
24. **Dinan L**. The Karlson Lecture. Phytoecdysteroids: what use are they? *Arch Insect Biochem Physiol* 72: 126-141, 2009.
25. **Dinan L**. Phytoecdysteroids: biological aspects. *Phytochemistry* 57: 325-339, 2001.
26. **Dinan L, and Lafont R**. Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals. *J Endocrinol* 191: 1-8, 2006.
27. **DK Mishra JF, MC Schmitz and RL Lieber**. resulting in short-term improvement but subsequent loss of muscle Anti-inflammatory medication after muscle injury. A treatment function. *J Bone Joint Surg Am* 77: 1510-1519, 1995.
28. **Dzhukharova M, Sakhibov A, Kasymov B, Syrov V, Takanaev A, and Saatov Z**. PHARMACOKINETICS OF ECDISTERONE IN THE EXPERIMENT. *KHIMIKO-FARMATSEVTICHESKII ZHURNAL* 21: 1163-1167, 1987.
29. **Goodman CA**. The role of mTORC1 in regulating protein synthesis and skeletal muscle mass in response to various mechanical stimuli. *Rev Physiol Biochem Pharmacol* 166: 43-95, 2014.
30. **Gordon L. Warren DALaRBA**. Measurement Tools Used in the Study of Eccentric Contraction–Induced Injury. *Sports Med* 27: 43-59, 1999.
31. **Gorelick-Feldman J, Cohick W, and Raskin I**. Ecdysteroids elicit a rapid Ca2+ flux leading to Akt activation and increased protein synthesis in skeletal muscle cells. *Steroids* 75: 632-637, 2010.
32. **Gorelick-Feldman J, Maclean D, Ilic N, Poulev A, Lila MA, Cheng D, and Raskin I**. Phytoecdysteroids increase protein synthesis in skeletal muscle cells. *J Agric Food Chem* 56: 3532-3537, 2008.
33. **Grebenok RJ, Ripa PV, and Adler JH**. Occurrence and levels of ecdysteroids in spinach. *Lipids* 26: 666-668, 1991.
34. **Hara K, Yonezawa K, Kozlowski MT, Sugimoto T, Andrabi K, Weng Q-P, Kasuga M, Nishimoto I, and Avruch J**. Regulation of eIF-4E BP1 Phosphorylation by mTOR. *Journal of Biological Chemistry* 272: 26457-26463, 1997.
35. **Hornberger TA, Mateja RD, Chin ER, Andrews JL, and Esser KA**. Aging does not alter the mechanosensitivity of the p38, p70S6k, and JNK2 signaling pathways in skeletal muscle. *J Appl Physiol (1985)* 98: 1562-1566, 2005.

36. **Hurme T, and Kalimo H**. Activation of myogenic precursor cells after muscle injury. *Medicine & Science in Sports & Exercise* 24: 197-205, 1992.
37. **Hurme T, Kalimo H, Lehto M, and Jarvinen M**. Healing of skeletal muscle injury: an ultrastructural and immunohistochemical study. *Medicine & Science in Sports & Exercise* 23: 801-810, 1991.
38. **Inoki K, Li Y, Zhu T, Wu J, and Guan K-L**. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* 4: 648-657, 2002.
39. **Israili ZH, and Lyoussi B**. Ethnopharmacology of the plants of genus Ajuga. *Pak J Pharm Sci* 22: 425-462, 2009.
40. **Jacobs BL, McNally RM, Kim KJ, Blanco R, Privett RE, You JS, and Hornberger TA**. Identification of mechanically regulated phosphorylation sites on tuberin (TSC2) that control mechanistic target of rapamycin (mTOR) signaling. *J Biol Chem* 2017.
41. **Jarvinen TA, Jarvinen TL, Kaariainen M, Kalimo H, and Jarvinen M**. Muscle injuries: biology and treatment. *Am J Sports Med* 33: 745-764, 2005.
42. **Khattak MJ, Ahmad T, Rehman R, Umer M, Hasan SH, and Ahmed M**. Muscle healing and nerve regeneration in a muscle contusion model in the rat. *J Bone Joint Surg Br* 92: 894-899, 2010.
43. **Kruger MJ, and Smith C**. Postcontusion polyphenol treatment alters inflammation and muscle regeneration. *Med Sci Sports Exerc* 44: 872-880, 2012.
44. **Kubica N, Bolster DR, Farrell PA, Kimball SR, and Jefferson LS**. Resistance Exercise Increases Muscle Protein Synthesis and Translation of Eukaryotic Initiation Factor 2Bϵ mRNA in a Mammalian Target of Rapamycin-dependent Manner. *Journal of Biological Chemistry* 280: 7570-7580, 2005.
45. **Kubo I, and Hanke FJ**. Chemical Methods for Isolating and Identifying Phytochemicals Biologically Active in Insects. In: *Insect-Plant Interactions*, edited by Miller JR, and Miller TA. New York, NY: Springer New York, 1986, p. 225-249.
46. **Kumpun S, Maria A, Crouzet S, Evrard-Todeschi N, Girault J-P, and Lafont R**. Ecdysteroids from Chenopodium quinoa Willd., an ancient Andean crop of high nutritional value. *Food Chemistry* 125: 1226-1234, 2011.
47. **Lawrence MM**. AJUGA TURKESTANICA AS A COUNTERMEASURE AGAINST SARCOPENIA AND DYNAPENIA. In: *Department of Health, Leisure and Exercise Science*Appalachian State University 2012.
48. **Lowe DA, Warren GL, Ingalls CP, Boorstein DB, and Armstrong RB**. Muscle function and protein metabolism after initiation of eccentric contraction-induced injury. *Journal of Applied Physiology* 79: 1260-1270, 1995.
49. **Massimino ML, Rapizzi E, Cantini M, Libera LD, Mazzoleni F, Arslan P, and Carraro U**. ED2+ Macrophages Increase Selectively Myoblast Proliferation in Muscle Cultures. *Biochemical and Biophysical Research Communications* 235: 754-759, 1997.
50. **Nozaki M, Li Y, Zhu J, Ambrosio F, Uehara K, Fu FH, and Huard J**. Improved muscle healing after contusion injury by the inhibitory effect of suramin on myostatin, a negative regulator of muscle growth. *Am J Sports Med* 36: 2354-2362, 2008.
51. **Ochieng CO, Ishola IO, Opiyo SA, Manguro LA, Owuor PO, and Wong KC**. Phytoecdysteroids from the stem bark of Vitex doniana and their anti-inflammatory effects. *Planta Med* 79: 52-59, 2013.
52. **Ogawa S, Nishimoto N, and Matsuda H**. Pharmacology of ecdysones in Vertebrates. In: *Invertebrate Endocrinology and Hormonal Heterophylly*Springer, 1974, p. 341-344.
53. **Pinniger GJ, Lavin T, and Bakker AJ**. Skeletal muscle weakness caused by carrageenan-induced inflammation. *Muscle Nerve* 46: 413-420, 2012.
54. **Proud CG**. mTOR-mediated regulation of translation factors by amino acids. *Biochemical and Biophysical Research Communications* 313: 429-436, 2004.

55. **Rahusen FTG**. Nonsteroidal Anti-inflammatory Drugs and Acetaminophen in the Treatment of an Acute Muscle Injury. *American Journal of Sports Medicine* 32: 1856-1859, 2004.
56. **Ramazanov NS**. Phytoecdysteroids and other biologically active compounds from plants of the genus Ajuga. *Chemistry of Natural Compounds* 41: 125, 2005.
57. **Rathbone CR, Wenke JC, Warren GL, and Armstrong RB**. Importance of satellite cells in the strength recovery after eccentric contraction-induced muscle injury. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 285: R1490-R1495, 2003.
58. **Sautour M, Canon F, Miyamoto T, Dongmo A, and Lacaille-Dubois MA**. A new ecdysteroid and other constituents from two Dioscorea species. *Biochemical Systematics and Ecology* 36: 559-563, 2008.
59. **Smith C, Kruger MJ, Smith RM, and Myburgh KH**. The inflammatory response to skeletal muscle injury: illuminating complexities. *Sports Med* 38: 947-969, 2008.
60. **Souza J, and Gottfried C**. Muscle injury: review of experimental models. *J Electromyogr Kinesiol* 23: 1253-1260, 2013.
61. **Sun Y, and Yasukawa K**. New anti-inflammatory ergostane-type ecdysteroids from the sclerotium of Polyporus umbellatus. *Bioorg Med Chem Lett* 18: 3417-3420, 2008.
62. **TIDBALL JG**. Inflammatory cell response to acute muscle injury. *Medicine & Science in Sports & Exercise* 27: 1022-1032, 1995.
63. **Tiidus PM**. Radical species in inflammation and overtraining. *Canadian Journal of Physiology and Pharmacology* 76: 533-538, 1998.
64. **Toth N, Szabo A, Kacsala P, Heger J, and Zador E**. 20-Hydroxyecdysone increases fiber size in a muscle-specific fashion in rat. *Phytomedicine* 15: 691-698, 2008.
65. **Tsivitse S**. Notch and Wnt signaling, physiological stimuli and postnatal myogenesis. *International journal of biological sciences* 6: 268-281, 2010.
66. **Walker DK, Dickinson JM, Timmerman KL, Drummond MJ, Reidy PT, Fry CS, Gundermann DM, and Rasmussen BB**. Exercise, Amino Acids and Aging in the Control of Human Muscle Protein Synthesis. *Medicine and Science in Sports and Exercise* 43: 2249-2258, 2011.
67. **Warren GL, Ingalls CP, Shah SJ, and Armstrong RB**. Uncoupling of in vivo torque production from EMG in mouse muscles injured by eccentric contractions. *The Journal of Physiology* 515: 609-619, 1999.
68. **Warren GL, Summan M, Gao X, Chapman R, Hulderman T, and Simeonova PP**. Mechanisms of skeletal muscle injury and repair revealed by gene expression studies in mouse models. *J Physiol* 582: 825-841, 2007.
69. **Wong TS, and Booth FW**. Protein metabolism in rat tibialis anterior muscle after stimulated chronic eccentric exercise. *Journal of Applied Physiology* 69: 1718-1724, 1990.
70. **Wyke SM, and Tisdale MJ**. Induction of protein degradation in skeletal muscle by a phorbol ester involves upregulation of the ubiquitin-proteasome proteolytic pathway. *Life Sci* 78: 2898-2910, 2006.
71. **Zanchi NE, and Lancha AH, Jr.** Mechanical stimuli of skeletal muscle: implications on mTOR/p70s6k and protein synthesis. *Eur J Appl Physiol* 102: 253-263, 2008.

Vita

Tyler Thomas Rice was born in Oil City, Pennsylvania, to Thomas and Jennifer Rice. He graduated from Slippery Rock University in Slippery Rock, Pennsylvania, in 2015. He graduated with a B.S. in Exercise Science. The following fall he was admitted to Appalachian State University to pursue a M.S. degree. Tyler is currently pursuing his M.S. at Appalachian State University in Exercise Science and is projected to graduate in May 2017.