ABSTRACT

Effects of Lower- and Higher- Volume Resistance Exercise on Serum Testosterone and Skeletal Muscle Androgen Receptor Content in Men: Subsequent Effects on the mRNA Expression of Insulin-Like Growth Factor Peptide and Myostatin in Skeletal Muscle

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Testosterone is the primary sex steroid hormone within males. Testosterone effects are ubiquitous and are categorized as either anabolic or androgenic. The androgen receptor is a specific nuclear hormone receptor through which testosterone elicits its effects. Specifically, skeletal muscle has androgen receptors present and is responsive to testosterone. The resulting activation of testosterone–androgen receptor–DNA binding increases muscle protein synthesis and reduces muscle protein breakdown. In addition, the testosterone level can alter the expression of insulin–like growth factor and myostatin which are key regulators of muscle protein balance. Higher-intensity resistance exercise in non-resistance-trained participants is known to increase endogenous serum testosterone levels. However, using resistance-trained participants the purpose of this study was to examine whether elevations in serum testosterone occur in response to a higher-intensity of resistance-exercise, and if this increase induces elevations in skeletal muscle testosterone, 5α-dihydrotestosterone (DHT), androgen receptor mRNA and protein content as well as possible interactions between muscle IGF-1 and myostatin

mRNA expression. In a randomized cross-over design, venous blood was obtained in male participants immediately before and after, 30 minutes, 1 hour, 2 hours, 3 hours, and 24 hours after a single bout of resistance exercise. Muscle samples were obtained immediately before and after, 3 hours, 24 hours after exercise. Exercise bouts consisted of an upper-lower body (higher-volume) and lower-body (lower-volume) protocol. Each exercise bout was separated by one week of rest. Statistical analyses were performed by separate 2 x 7 and 2 x 4 (Session x Test) factorial analyses of variance (ANOVA) with repeated measures. The exercise protocol employed has previously been demonstrated to significantly increase serum testosterone. However, this study did not produce such a response. Neither exercise protocol significantly increased serum total or free testosterone ($p > 0.05$). Also, no changes were observed for muscle testosterone, DHT, androgen receptor mRNA expression or protein content ($p > 0.05$). In addition, no alterations in muscle IGF-1 or myostain were observed ($p > 0.05$). It was concluded that higher-intensity resistance exercise of the upper-body performed immediately prior to lower-body resistance exercise does not result in a significant elevation in testosterone in resistance-trained participants.

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LIST OF ABBREVIATIONS

AMP – Adenosine Monophosphate
AR – Androgen Receptor
ARE – Androgen Response Element
BIA – Bioelectrical Impedance Analysis
BIS – Bioimpedance Spectroscopy
BP – Base Pair (bp)
CNS – Central Nervous System
DBD – DNA Binding Domain
cDNA – Complementary Deoxyribonucleic Acid
DEXA – Dual Energy X-ray Absorptiometry
DHT – 5α-dihydrotestosterone
DNA – Deoxyribonucleic Acid
ELISA – Enzyme-linked Immunosorbent Assay
FSH – Follicle Stimulating Hormone
GnRH – Gonadotropin-Releasing Hormone
HPG – Hypothalamic Pituitary Gonadal
HRP – Horseradish Peroxidase
HSP – Heat Shock Protein
HV – High Volume
LBD – Ligand Binding Domain
IGF – Insulin-like Growth Factor
LH – Luteinizing Hormone
LV – Low Volume
MGF – Mechano Growth Factor
mRNA – Messenger Ribonucleic Acid
NCBI – National Center for Biotechnology Information
NTD – N-Terminal Regulatory Domain
OD – Optical Density
PCR – Polymerase Chain Reaction
RNA – Ribonucleic Acid
SHBG – Sex Hormone Binding Globulin
TMB – 3,3’,5’5-Tetramethylbenzidine
USG – Urine Specific Gravity
1-RM – 1-repetition maximum

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DEDICATION

In memory of
Ryan Patrick Spillane
&
Chair of the HHPR Department Dr. Rafer S. Lutz

CHAPTER ONE

Introduction

Testosterone is the primary androgenic sex steroid hormone within males. Production is tightly regulated by the hypothalamic-pituitary-gonadal (HPG) axis, wherein the Leydig cells of the testes account for 95% of the testosterone produced within adult males (Kicman, 2010; Rommerts, E. Nieschlag, & Nieschlag, 2004). Testosterone effects are ubiquitous and are categorized as either anabolic (i.e. growth) or androgenic (i.e. male sex characteristics). The primary anabolic effect of testosterone is via skeletal muscle adaptation by promoting increased muscle protein synthesis. Testosterone's ability to promote muscle protein synthesis depends on numerous biochemical and molecular interactions. Specifically, testosterone will bind to a specific nuclear hormone receptor (i.e. androgen receptor) which migrates into the nucleus binding to the androgen response element on DNA resulting in an up-regulation of gene expression (Kicman, 2010). The ability for testosterone to bind to the androgen receptor is one critical factor controlling the rate of protein synthesis. Fluctuations in serum testosterone levels have also shown to alter androgen receptor content within skeletal muscle (Bamman et al., 2001; W. J. Lee, McClung, Hand, & Carson, 2003; Willoughby & Taylor, 2004).

Insulin-like growth factor 1 (IGF-1) is a polypeptide hormone which also plays a critical role in skeletal muscle growth. Different variants or isoforms of IGF-1 are known to exist. IGF-1 functions both systemically and locally within skeletal muscle (Denley, Cosgrove, Booker, Wallace, & Forbes, 2005). Locally produced IGF-1 is known as IGF-

1Ec or MGF (mechano-growth factor) (McKay, O'Reilly, Phillips, Tarnopolsky, & Parise, 2008). The IGF-1 gene promoter region has shown to have an androgen response element indicating IGF-1 expression can be altered by testosterone (Wu et al., 2007). The expression of IGF-1 has shown to be modified by circulating serum testosterone levels (Inder, Jang, Obeyesekere, & Alford, 2010). This indicates testosterone not only has a direct effect on skeletal muscle growth, but also acts to promote growth through up-regulation of the locally-expressed IGF-1 peptide.

Another key regulator of skeletal muscle adaptation is myostatin. Myostatin is a member of the transforming growth factor-β superfamily and is a negative regulator of skeletal muscle (McPherron, Lawler, & Lee, 1997; Schuelke et al., 2004). The androgen response element has also shown to be present in the promoter sequence of the myostatin gene (Ma et al., 2001). Testosterone is a repressor of myostatin gene expression; therefore, upon binding of testosterone to the androgen response element within the myostatin gene, expression is decreased. An elevation in myostatin has also shown to reduce the activity of the androgen receptor (Siriett et al., 2006). This indicates that testosterone/myostatin interactions are key regulators to skeletal muscle growth and degradation.

Resistance exercises in both acute (i.e., single bouts) and chronic (i.e., longer-term training programs) scenarios are known to increase muscle protein synthesis, resulting in a promotion of skeletal muscle hypertrophy (Abernethy, Jurimae, Logan, Taylor, & Thayer, 1994; Fitts & Widrick, 1996). Specifically, exercise can alter the hormonal environment both systemically and locally. Resistance exercise has shown the capacity to stimulate an increase in testosterone and IGF levels depending on the protocol

(W. J. Kraemer & Ratamess, 2005; Spiering et al., 2008; Vingren et al., 2010). Reductions in myostatin levels have also been shown during both acute and chronic exercise (Favier, Benoit, & Freyssenet, 2008; Louis, Raue, Yang, Jemiolo, & Trappe, 2007). In addition, the androgen receptor protein expression within skeletal muscle has shown to be responsive to resistance exercise (Vingren et al., 2009; Willoughby & Taylor, 2004).

Testosterone/androgen receptor interactions together with IGF and myostatin have a profound effect on skeletal muscle adaptation, ultimately affecting muscle hypertrophy. A transient increase of endogenous circulating serum testosterone levels with resistance exercise is known to occur. The impact of this elevation of serum testosterone seen during resistance training to increase testosterone levels within the skeletal muscle in addition to the interaction with the androgen receptor, IGF and myostatin still needs to be determined.

Problem Statement

How does an endogenous elevation in testosterone resulting from a single resistance exercise bout alter: 1) androgen levels within the blood and muscle tissue, androgen receptor mRNA expression and protein content; 2) muscle specific IGF-1 mRNA expression; and 3) muscle myostatin mRNA expression.

Purpose of Study

Compared to the increase in serum testosterone resulting from a lower-body resistance exercise protocol, when upper-body resistance exercise is then performed immediately prior to lower-body resistance exercise the serum testosterone increase has

been shown to be greater (Spiering et al., 2008). Therefore, the purpose of this was to study was to examine the effect of an elevation in endogenous testosterone levels immediately before and after, 30 minutes, 1 hour, 2 hours, 3 hours, and 24 hours after a single bout of resistance exercise. Based on previous research (Spiering et al., 2008), higher-intensity resistance exercise of the upper-body performed immediately prior to lower-body resistance exercise should result in a significant elevation in blood testosterone. Specifically, the purpose of this study was to determine if elevated endogenous testosterone would result in an increase in muscle testosterone, DHT, androgen receptor mRNA and protein content. A secondary purpose was to evaluate the interaction between resistance exercise and elevated testosterone response on muscle IGF-1 mRNA expression. Thirdly, in order to determine androgen-responsiveness of the myostatin gene, the effect of testosterone and androgen receptor activity on myostatin mRNA expression was examined.

Hypotheses

H_1: Following the exercise bout involving higher-volume (upper- & lower-body) resistance exercise, a significant increase in serum testosterone will occur compared to the exercise bout involving lower-volume (lower-body) resistance exercise.

H_2: Following the exercise bout involving higher-volume (upper- & lower-body) resistance exercise, a significant increase in muscle testosterone will occur compared to the exercise bout involving lower-volume (lower-body) resistance exercise.

H_3: Following the exercise bout involving higher-volume (upper- & lower-body) resistance exercise, there will be a significant increase in muscle DHT compared to the exercise bout involving lower-volume (lower-body) resistance exercise.

H_4: Following the exercise bout involving higher-volume (upper- & lower-body) resistance exercise, a significant decrease in muscle myostatin mRNA expression will occur compared to the exercise bout involving lower-volume (lower-body) resistance exercise.

H_5: Following the exercise bout involving higher-volume (upper- & lower-body) resistance exercise, a significant increase in muscle IGF-1 mRNA expression will occur compared to the exercise bout involving lower-volume (lower-body) resistance exercise.

H_6: Following the exercise bout involving higher-volume (upper- & lower-body) resistance exercise, a significant increase in androgen receptor mRNA expression will occur compared to the exercise bout involving lower-volume (lower-body) resistance exercise.

H_7: Following the exercise bout involving higher-volume (upper- & lower-body) resistance exercise, a significant increase in androgen receptor protein content will occur compared to the exercise bout involving lower-volume (lower-body) resistance exercise.

Delimitations

- Ten apparently healthy males between the ages of 18-30 who had consistent resistance training (3x/week) for at least one year prior to the study.

- Participants were recruited from Baylor University and within the surrounding Waco, Tx area by flyers and online advertisements.

- Participant were excluded from the study if prior ingestion (within 6 months) of any dietary supplement or pharmaceutical aid used as a potential ergogenic aids.

- All participants were considered low risk for cardiovascular disease, with no contraindication to exercise as outlined by the American College of Sports Medicine (ACSM).

- Participants were in a euhydrated state prior to participation in both exercise bouts.

- All participants were tested at the Baylor Laboratory for Exercise Science and Technology (BLEST) and Exercise Nutritional Biochemical Laboratory (EBNL) in accordance with Helsinki Code after signed university approved informed consent documents.

Limitations

- The study utilized a purposeful sampling of those individuals who were within the city of Waco, as well as a small sample size (n=10), external validity to the greater population of resistance trained males (18-30 y) may be reduced; although, it is unlikely to be a significant concern

- Participants were expected to maximally exert themselves during both the upper body/lower body and lower body only resistance exercise bouts

- Each participant had inherent circadian rhythms that would likely altered hormonal levels throughout the day; this variation was minimized by testing during the morning (am) hours for each participant

- Since the biopsy procedure may have caused inflammation to the site of extraction; to minimize any possible stress response, additional samples were taken from incision 0.5cm medial or lateral to the original biopsy site

Assumptions

- All laboratory equipment was functioning properly with validity and reliability measurements being established. Proper calibration and the use of trained research staff minimized any potential for errors.

- All participants followed the guidelines provided and performed the exercises at maximal effort during the testing sessions

- All participants were considered truthful in their training status: consistent resistance training (3x/week) for at least one year prior to the study.

- All participants were to arrive at each testing session in a fasted state (>8 hours)

- All participants were to arrive with adequate sleep (7-8 hours) before each of the testing sessions

Definitions

- Aromatization - A reaction that converts a substance into an aromatic compound, specifically conversion of androgens into estrogens (Bruce Alberts, 2008; Kicman, 2010)

- AR – Androgen Receptor, type of nuclear receptor that is activated by binding of androgenic hormones testosterone or 5α-dihydrotestosterone (Kicman, 2010) (Bruce Alberts, 2008)

- ARE – Androgen Response Element, specific genomic site to which androgen receptor binds and modulates transcription of nearby genes (Bruce Alberts, 2008; Kicman, 2010)

- BIA – Bioelectrical Impedance Analysis, method to measure body composition specifically body water through the use of a low level electrical current by measuring the resistance to the current (Lukaski, Johnson, Bolonchuk, & Lykken, 1985)

- BIS – Bioelectrical Impedance Spectroscopy, method to measure body composition specifically body water through the use of multiple low level frequencies (Armstrong, 2007)

- Cortisol – main glucocorticoid form in humans, catabolic hormone secreted from the adrenal cortex in response to physical and/or psychological stress (Brownlee, Moore, & Hackney, 2005)

- DEXA- Dual Energy X-ray Absorptiometry, imaging technique that uses two low-dose x-ray beams to measure the density of specific tissues (bone mineral, lean tissue, adipose tissue) (Van Loan & Mayclin, 1992)

- DHT - 5α-dihydrotestosterone, derivative of testosterone having androgenic and anabolic activities. Responsible for male primary sex characteristics (Bruce Alberts, 2008; Kicman, 2010)

- DNA – Deoxyribonucleic Acid, is a nucleic acid containing the genetic instructions used in the development and functioning of all known living organisms (Bruce Alberts, 2008)

- Eugonadal – Body produces the correct amount of testosterone to fulfill normal bodily functions (Kicman, 2010)

- Forced Repetition – After a trainee has achieved a momentary concentric failure (i.e. a set until exhaustion has been performed), a training partner will assist by lifting or pushing the load just enough to allow the trainee to complete three to four additional repetitions (Juha P Ahtiainen, Pakarinen, Kraemer, & Häkkinen, 2003; S. F. a. W. Kraemer, 2004)

- Free Testosterone – Testosterone not bound to binding proteins (Bruce Alberts, 2008; Kicman, 2010)

- IGF-1 – Insulin Like Growth Factor -1, polypeptides hormone similar to insulin which stimulates protein synthesis (Bruce Alberts, 2008; Denley, et al., 2005)

- MGF – Mechano Growth Factor also known as IGF-1Ec, splice variant of insulin like growth factor which is produced in response to mechanical stress within skeletal muscle (Barton, 2006; Hameed, Orrell, Cobbold, Goldspink, & Harridge, 2003)

- mRNA- Messenger Ribonucleic Acid, A coding molecule that provides genetic information for the cell to produce new proteins (Bruce Alberts, 2008)

- Myofibrillar Protein – a muscle fibril, one of the slender threads of a muscle fiber, composed of numerous myofilaments (Bruce Alberts, 2008)

- Myostatin – Member of the transforming growth factor-β (TGF-β) superfamily, functions as negative regulator of skeletal muscle (McPherron, et al., 1997)

- Promoter Sequence – A regulatory DNA sequence that initiates the expression of a gene (Bruce Alberts, 2008)

- SHBG – Sex hormone binding globulin, glycoprotein that binds to testosterone (Bruce Alberts, 2008; Kicman, 2010)

- Testosterone – androgenic (Steroid) hormone, primarily produced within the Leydig's cells of the testis in men (Bruce Alberts, 2008; Kicman, 2010)

- 1-RM – 1 repetition maximum, the maximum amount of weight one can lift in a single repetition for a given exercise (S. F. a. W. Kraemer, 2004)

CHAPTER TWO

Literature Review

Testosterone Production

The major circulating androgen within males is testosterone (17β-hydroxy-4-androstenen-3-one), which is a 0.288 kD C_{19} steroid hormone produced from cholesterol (Rommerts, et al., 2004; Vingren, et al., 2010). Cholesterol is utilized in the formation of testosterone by a series of enzymatic reactions primarily within the Leydig cells of the testes (Cigorraga, Dufau, & Catt, 1978; Mendelson, Dufau, & Catt, 1975). The testes account for greater than 95% of the testosterone production within an adult male; this accounts for roughly 3 -7 mg per day (Kicman, 2010; Rommerts, et al., 2004). However, testosterone can also be produced in small quantities within the zona reticularis of the adrenal cortex, along with brain and nervous system cells (Baulieu, 1997; King et al., 2002; Marouliss & Triantafillidis, 2006). Two major classes of enzymes (cytochrome P450 and hydroxysteroid dehydrogenase) are required for the biosynthesis of testosterone from cholesterol within the gonads and adrenal gland. Specifically, a cholesterol side chain is catalyzed by enzyme CYP11A to yield the C_{21} steroid pregnenolone. This is the rate limiting step in testosterone biosynthesis. Pregnenolone can also be catalyzed into progesterone by enzymatic reaction of 3βHSD. Pregnenolone and/or progesterone will further yield either 17α-hydroxypregnenolone or 17α-hydroxyprogesterone by enzymatic reaction involving CYP17. Additional cleavage will take place yielding the C_{19} steroids dehydroepiandrosterone (DHEA) or androstenedione. Within the gonads,

androstenedione is catalyzed into the active steroid hormone testosterone via 17HSD3 (Miller & Auchus, 2011; Payne & Hales, 2004).

Testosterone Regulation HPG Axis

Testosterone production is tightly regulated through the interaction of the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamus is innervated by the central nervous system (CNS) in which three neuropeptides (kisspeptin, neurokinin B, dynorphin) control the production and secretion of gonadotropin-releasing hormone (GnRH) (Lehman, Coolen, & Goodman, 2010). GnRH is released from the hypothalamus into the hypophyseal portal circulation wherein GnRH subsequently binds to receptors on gonadotropes within the pituitary (Kaiser, Sabbagh, Katzenellenbogen, Conn, & Chin, 1995; Veldhuis et al., 2009). Gonadotropin stimulation results in the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary (Kaiser, et al., 1995; Keenan & Veldhuis, 1998; Veldhuis, et al., 2009). GnRH is released as pulsatile bursts from the hypothalamus (Keenan & Veldhuis, 1998; Veldhuis, et al., 2009). The consequence of this pulsatility results is downstream diurnal variation in testosterone production. Testosterone levels have also shown to be 30-35% higher during the morning hours (0800–1200 hours) compared to mid-to-late afternoon (Brambilla, Matsumoto, Araujo, & McKinlay, 2009). LH and FSH, once released from the anterior pituitary, will enter systemic circulation and act upon the gonads. LH will bind to G-protein LH receptors within the Leydig cells resulting in an activation of cyclic AMP. Activation of the receptors results in the stimulation of testosterone production (Cigorraga, et al., 1978; Mendelson, et al., 1975; Miller & Auchus, 2011). FSH stimulates the production of sex hormone binding globulin (SHBG), but does not directly

result in testosterone production within males (Rosner, Hryb, Kahn, Nakhla, & Romas, 2010). Testosterone, once synthesized is not stored, but rather rapidly released following production. Regulation of the HPG axis is tightly controlled by stimulation of the pituitary by the CNS or through a negative feedback loop. Stimulation of the CNS results in GnRH release, which subsequently leads to testosterone production within the gonads. Elevation in testosterone levels will result in a negative feedback action upon the hypothalamus, thereby reducing the release of GnRH. The reduction in the release of GnRH results in a diminished release of LH and FSH in the anterior pituitary, which inhibits testosterone production within the gonads (Cigorraga, et al., 1978; Sharma et al., 2012; Veldhuis, et al., 2009).

Testosterone Transport

Testosterone is a lipid-based steroid hormone that is not readily transported within the blood due to the hydrophobic nature of the hormone. Specific hydrophilic proteins are required to circulate testosterone within the blood (Burton & Westphal, 1972). Total blood testosterone levels are comprised of testosterone bound to these proteins along with a small fraction of unbound testosterone. Testosterone can be bound to SHBG, which accounts for 44% to 60% of total serum testosterone levels (Heinlein & Chang, 2002; Kicman, 2010; Rommerts, et al., 2004; Vingren, et al., 2010). The remaining testosterone is either weakly bound to albumin or unbound (free), which accounts for roughly 2% of total serum testosterone levels (Hammond, Nisker, Jones, & Siiteri, 1980; Rommerts, et al., 2004; Vingren, et al., 2010). Testosterone that is bound to SHBG is not available for transport into target tissues, thus inhibiting any possible interaction with the androgen receptor (Pardridge, 1986). The bioavailability of testosterone bound to albumin has

shown to be high, with approximately 55% of albumin-bound testosterone being able to enter the target tissues (Manni et al., 1985). Free testosterone is the most biologically active form of testosterone allowing for the highest androgen receptor binding potential (Rommerts, et al., 2004; Vingren, et al., 2010).

Testosterone Metabolism

There are several naturally occurring endogenous androgens. The most active androgens are testosterone and 5α-dihydrotestosterone (DHT). The oxidation of 17β-hydroxyl group and/or reduction of 3-oxo group of testosterone and/or DHT can yield other androgens [5α-androstanediol, Androstenedione, Epitestosterone, Dehydroepiandrosteone (DHEA), Androsterone, Etiocholanolone] (Kicman, 2010). The result of oxidation is either a reduction or a total loss of androgenic activity. The levels of androgen within the body are determined by the rate of synthesis and degradation. The range of serum testosterone concentration in eugonadal men is 3-10 ng mL^{-1} (Kicman, 2010). The metabolism (aromatization) of testosterone can take place in various tissue (i.e. placenta, ovary, testis, adipose tissue, liver, hair follicles, brain, blood vessels, bone, and cartilage) by cytochrome P450 enzyme (Czajka-Oraniec & Simpson, 2010; Rommerts, et al., 2004). However, the main site of androgen metabolism takes place within the liver (Kicman, 2010). The aromatization of circulating androgens account for 85% of 17 β-estradiol and 95% of the estrone produced in males (Czajka-Oraniec & Simpson, 2010; Simpson, 2003; Simpson et al., 2002). Testosterone aromatization into estrogen takes place to a large extent within adipose tissue. However, some estrogen production also takes place within skeletal muscle tissue (Larionov et al., 2003). Testosterone is also aromatized into DHT by 5α-reductase, which exists as either type 1

and 2 isoforms (Simpson, 2003; Thigpen et al., 1993). These NADPH-dependent enzymes, which are located in the microsomes of the cell, reduce the bonds of C_{19} and C_{21} steroids (Imperato-McGinley & Zhu, 2002). As the product of testosterone aromatization, DHT is able to bind to the same intracellular receptor as testosterone, yet DHT has a higher affinity for the androgen receptor (Imperato-McGinley & Zhu, 2002; Kicman, 2010).

Biological Effects of Testosterone

The traditional hypothesis of testosterone entry into the cell states that free testosterone or testosterone released from binding proteins can traverse the plasma membrane due to lipid solubility of the steroid hormone (Adams, 2005; Hammes et al., 2005; Kicman, 2010). However, research in cultured cells conducted by Hammes *et al. 2005* demonstrated that SHBG can bind to megalin (low-density lipoprotein receptor-related protein). SHBG binding to megalin internalizes SHBG so that it can by degraded by lysosomes, thereby resulting in the release of steroids within the cellular environment. While this process still needs to be further elucidated, the traditional process allows for rapid entry of testosterone through the plasma membrane. Once testosterone translocates inside the cell it has two fates. It can bind to a specific nuclear receptor or can be converted into 5α-dihydrotestosterone (DHT) by 5α-reductase which will then bind to the same nuclear receptor (Kicman, 2010). Specifically, within skeletal muscle little activity of 5α-reductase has been shown compared to other tissues such as the skin and prostate tissues (Hsiao, Thin, Lin, & Chang, 2000; Thigpen, et al., 1993; Zouboulis, Chen, Thornton, Qin, & Rosenfield, 2007). This indicates that DHT levels would be low within skeletal muscle leading to testosterone being the primary androgen binding to the nuclear

receptor. Endogenous androgen will mediate physiological effects upon skeletal muscle by altering androgen receptor expression and intracellular metabolism, which occurs along genomic and non-genomic pathways (Kicman, 2010).

Androgen Receptor

The androgen receptor is a member of ligand activated nuclear hormone receptor super family (Kicman, 2010; Li & Al-Azzawi, 2009). The androgen receptor contains an N-terminal regulatory domain (NTD), DNA binding domain (DBD) (Nindl et al., 2001), variable hinge region (H), a ligand binding domain (LBD), and two transcriptional activation domains AF-1 and AF-2 (Askew, Minges, Hnat, & Wilson, 2012; Kicman, 2010; Li & Al-Azzawi, 2009). Two isoforms of the androgen receptor are present. Androgen receptor A is a 87 kDa isoform with a reduced NTD region while the Androgen receptor B isoform NTD region is full length 110 kDa (Li & Al-Azzawi, 2009; Wilson & McPhaul, 1994). Androgen receptor B is the predominate isoform in a variety of both fetal and adult human tissues (Wilson & McPhaul, 1996). While, androgen receptor A may be expressed in the same tissues, it is not able to mediate all the effects compared to androgen receptor B upon androgen binding (Wilson & McPhaul, 1994). Androgen receptor expression has shown to be responsive to elevations in testosterone levels. Ferrando *et al.* (2002) have shown during pharmaceutical treatment with intramuscular testosterone injection in elderly men, an up regulation of the AR after one month. However, after six months of use the AR protein returned to baseline levels. Other have shown similar results with acute increases in testosterone leading to an up-regulation of the AR, but a return to baseline after extended exposure (Carson, Lee, McClung, & Hand, 2002; Ferrando, Sheffield-Moore, Wolf, Herndon, & Wolfe, 2001;

Kadi, Eriksson, Holmner, & Thornell, 1999; W. J. Lee, Thompson, McClung, & Carson, 2003). Shinha-Hikim *et al.* (2004) demonstrated that cycling on and off 600 mg of testosterone enanthate for 20 weeks can lead to a long term increase in AR protein (Sinha-Hikim, Taylor, Gonzalez-Cadavid, Zheng, & Bhasin, 2004).

Testosterone – AR Interaction

Once testosterone translocates inside the cell, the steroid hormone will bind to the AR. The AR is sequestered by specific chaperone proteins known as heat shock proteins (HSPs). Specifically HSP90, HSP70, and HSP56 are bound to the AR. When the androgens, testosterone or DHT, bind to the AR this results in a transformation of the receptor in which HSPs are dissociated allowing the testosterone-AR bound complex to become active (Gelmann, 2002; Veldscholte et al., 1992). DHT has a greater binding affinity for the AR compared to testosterone (George & Noble, 1984; Kicman, 2008, 2010). The bound active complex will translocate from the cytosol into the nuclei where it will bind to a specific area on DNA, classified as the androgenic response element (ARE) (Bennett, Gardiner, Hooper, Johnson, & Gobe, 2010). The DNA binding domain on the AR contains two zinc finger-like motifs that allow for insertion in a groove within the ARE (Claessens et al., 2001; Helsen et al., 2012). Activation of the androgen receptor by testosterone or DHT with subsequent binding to the ARE results in the expression of targeted genes (Maurer et al., 2001). This is considered to be the classic genomic effect of androgen actions. Attachment of the testosterone/AR complex to the ARE on DNA triggers formation of transcription complexes which activates gene sequences altering the transcription and/or translation of that gene (Kicman, 2010). However, testosterone can also act in more rapid non-genomic effects. Androgens can

potentially bind to specific binding sites on specific molecules in the absence of the androgen receptor. They may also bind to a trans-membrane G-protein coupled receptors or alter changes in membrane fluidity. These changes can be mediated through increases in intracellular calcium or activation of signaling cascades such as mitogen-activated protein kinase (MAPK) and phosphatidyl-inositol-3 kinase (PI-3K) (Michels & Hoppe, 2008).

Insulin-Like Growth Factor

Insulin-like growth factors (IGF) are small polypeptides which are also known as somatomedins. There are two classes of IGF's known IGF-1 and IGF-2. IGF-1 is a 70 amino acid polypeptide and IGF-2 is a 67 amino acid polypeptide, and each can be classified into four domains of B,C, A, and D (Denley, et al., 2005). While IGF-1 and 2 have a similar structure to insulin, they act in distinct ways. Serum IGF-1 levels are regulated by the endocrine system; growth hormone is released by the pituitary which acts upon the liver resulting in synthesis and systemic release of IGF-1. IGF-1 synthesis typically takes 8 to 29 hours once growth hormone stimulates liver production. The majority of circulating IGF comes from the liver, accounting for 75% of the total IGF in circulation (Barton, 2006; Schwander, Hauri, Zapf, & Froesch, 1983). IGF enters into the systemic circulation were it can bind to the appropriate target tissue. Within the blood, 99% of IGF can be bound to specific binding proteins. These binding proteins are classified 1 through 6 (Denley, et al., 2005). Free IGF and IGF bound to the binding proteins are then able to bind to the insulin-like growth factor-1 receptor (IGF-1R). Various cells (muscle, brain, kidney, and adipose tissue) can also manufacture their own IGF. Adipose tissue has a relative high concentration of IGF compared to skeletal muscle

(Barton, 2006). Different isoforms of IGF will have identical 70 amino acid proteins and contain the B, C, A, and D domains. Yet, splice variants can be seen within the E-peptide. Within skeletal muscle, a splice variant of IGF is known as IGF-1Ec or MGF (mechano growth factor) which is named due to the ability to respond to mechanical stress (Barton, 2006; Hameed, et al., 2003; McKay, et al., 2008). Two AREs are found within the upstream regulatory promoter region of the IGF-1 gene in order to activate IGF-1 expression (Wu, et al., 2007). Testosterone may play a role in IGF-1 gene expression. This has been demonstrated within older men (>60 years) who received testosterone enanthate for six months, in which IGF-1 protein expression was elevated throughout the six month trial (Ferrando et al., 2002). Men (58 ± 3 years) who were given a drug to reduce testosterone levels resulted in a significant reduction in skeletal muscle androgen receptor and IGF-1 mRNA expression (Inder, et al., 2010).

Myostatin

Myostatin is a member of the transforming growth factor-β (TGF-β) superfamily, also known as growth/differentiation factor-8 (GDF-8), which is expressed in both developing and adult skeletal muscle cells (S. J. Lee & McPherron, 2001; McPherron, et al., 1997). The function of myostatin is as a negative regulator of muscle mass (Bogdanovich et al., 2002; Schuelke, et al., 2004; Whittemore et al., 2003). Myostatin regulation has shown to be regulated by various factors [proteases BMP-1/TLD family (BMP-1, TLD, TLL-1, TLL-2)], follistatin, FSTL-3, growth and differentiation factor-associated serum protein (GASP-1, GASP-2), latent TGF-β Binding Protein-3 (LTBP-3), Decorin, Titin-Cap, hSGT) (S. J. Lee, 2010). Testosterone is also thought to be a regulator of myostatin. Mendler *et al.* (2005) showed a significant up-regulation in

myostatin protein in castrated rats, yet when treated with testosterone a reduction in myostatin protein was observed (Mendler, Baka, Kovacs-Simon, & Dux, 2007). The myostatin gene has shown to contain an ARE in the promoter sequence allowing for regulation by androgen binding (Ma, et al., 2001). Singh *et al.* (2009) demonstrated that testosterone up-regulates the expression of follstatin, which results in an inhibitory effect upon the TGF-β/Smad signaling pathway. The consequence of this reduction in a myostatin signaling is an increased myogenic differentiation (Singh et al., 2009). Elevated myostatin levels have shown to down-regulate androgen receptor associated protein-70 (ARA70), thereby reducing the activity of the AR (Siriett, et al., 2006). Myostatin is known to inhibit the IGF-1- mediated myoblast differentiation and myotube size through an inhibition of Akt/mTOR/p70S6K signaling pathway (Morissette, Cook, Buranasombati, Rosenberg, & Rosenzweig, 2009; Trendelenburg et al., 2009). Lakshman *et al.* (2009) gave young men (18-35 years) monthly injections of a GnRH agonist to suppress endogenous testosterone production. The subjects then were provided weekly injection of testosterone enanthate at 25, 50, 125, 300 mg for 20 weeks. Myostatin levels significantly increased at day 56 of the treatment, but returned to baseline levels by the end of the study. Myostatin levels were positively correlated with the change in both total and free testosterone at day 56 (Lakshman et al., 2009). It was suggested that myostatin may be a counter-regulatory mechanism to control muscle size. Myostatin is produced and secreted into plasma by skeletal muscle. As the result of increased testosterone levels, an increase in muscle mass results. This increase in muscle mass results in an elevation of myostatin secretion to possibly restrain uncontrolled skeletal muscle growth (S. J. Lee, 2004).

Exercise Hormonal/Molecular Responses

Testosterone

Resistance exercise has shown to elicit a testosterone response in numerous studies (W. J. Kraemer et al., 1991; W. J. Kraemer, Hakkinen, et al., 1999; W. J. Kraemer et al., 1990; Roberts, Dalbo, Hassell, & Kerksick, 2009; Spiering, et al., 2008). The testosterone response seen within resistance exercise depends on numerous factors. Specifically, younger age men (20-30 years) will have a greater response compared to adolescent (14-18 years), middle age (38-53 years), and older (≥59 years) men and women (Vingren, et al., 2010). The type of exercise bout will also alter the testosterone response. Exercise bouts will need to have high-intensity (load) (85%-95%) of one repetition max and meet a minimum threshold. A moderate to high-volume (set x number of reps x intensity) is also required. This can be achieved by changing the number of sets or number of exercises. The choice of exercises that utilize large muscle groups (i.e. power clean, squats, and dead lifts) will elicit the greatest response. Performing resistance exercise in order with large muscle groups first, along with utilizing short rest periods (30-60 seconds), will also result in the largest testosterone response (W. J. Kraemer, et al., 1990; Spiering, et al., 2008; Vingren, et al., 2010).

Insulin-Like Growth Factor

Systemic IGF-1 has also shown to increase in both acute and chronic resistance exercise bouts in some studies (Haddad & Adams, 2002; W. J. Kraemer & Ratamess, 2005; Spillane et al., 2011). However, others have shown no significant increase in serum

IGF-1 levels in both young (30 years) and old (62 years) with a 10-week training program (W. J. Kraemer, Hakkinen, et al., 1999). However, Kraemer *et al.* (1999) did show a significant increase in total and free testosterone after the exercise bout. Psilander *et al.* (2003) showed a 44% decrease in IGF-1E abc isoform mRNA levels one and six hours after an acute resistance exercise bout. Yet, 24 hours post-exercise an elevation was shown (Psilander, Damsgaard, & Pilegaard, 2003). The upstream promoter of IGF-1 has two identified AREs, which indicate testosterone has the ability to up-regulate IGF-1 gene expression (Wu, et al., 2007). Mauras *et al.* (1998) examined the effect of testosterone suppression for 10 weeks in young men (23.2 ± .5 years). The decrease in testosterone levels were correlated with a significant decrease in muscle IGF-1 mRNA expression (Mauras, Hayes et al. 1998). Bamman *et al.* (2001) showed a 62% increase in muscle IGF-1 mRNA after eccentric squat exercise. Yet, no change in serum testosterone or IGF-1 levels were observed (Bamman, et al., 2001). This may indicate that a transient increase in testosterone does not necessarily mean transactivation of the IGF-1 gene in all cases. Additional research is needed to further elucidate the mechanisms by which IGF-1 and testosterone interact.

Myostatin

Myostatin expression has shown to be altered during acute resistance exercise bouts. In young (29 ± 2 years) and old (70 ± 2 years) men, myostatin mRNA expression was significantly reduced by (~50%) from baseline levels at both three and six hours post-exercise in both groups (Drummond et al., 2009). However, the researchers used a combination of an acute resistance exercise stimulus (leg extensions) along with ingestion of an essential amino acid supplement. Louis *et al.* (2007) showed myostatin

mRNA was decreased 6.3-fold up to 24 hours post-exercise (Louis, et al., 2007). Kim *et al.* (2005) showed similar results with a reduction in myostatin mRNA 24 hours after resistance exercise in both young (20-35 your) males (-56%), females (-48%), and older (60-75 year) males (-40%) (Kim, Cross, & Bamman, 2005). Hulmi *et al.* (2008) has also shown a significant decrease in myostatin mRNA 48 hours after an acute bout of heavy resistance exercise in older men (57-72 year) (Hulmi, Kovanen, Lisko, Selanne, & Mero, 2008). In both young (32 ±7 year) and elderly (72 ± 5 year) males, myostatin mRNA has also shown to decrease up to 72 hours after an acute exercise bout by 52% and 42%, respectively (Dennis et al., 2008). Thus, acute resistance exercise appears to inhibit myostatin mRNA expression.

Androgen Receptor

The AR mRNA and protein content has shown to be altered after resistance exercise. As a result of a single bout of resistance exercise, the AR has shown to be down-regulated immediately post-exercise (Vingren, et al., 2010). Ratamess *et al.* (2005) showed a 46% down-regulation of the AR immediately following 6 sets of 10 repetition squat exercises (Ratamess et al., 2005). Vingren *et al.* (2009) showed similar results with a down-regulation 70 minutes post-exercise with a similar exercise protocol (Vingren, et al., 2009). However, when AR mRNA was measured 48 hours after an acute exercise bout, a significant increase in mRNA has been shown (Hulmi et al., 2008). Spiering *et al.* (2009) showed a similar results with a significant increase in AR content 3 hours post-exercise (Spiering et al., 2009). Willoughby and Taylor (2004) have also shown a significant increase in AR mRNA and protein content 48 hours post-exercise after sequential bouts (Willoughby & Taylor, 2004). This indicates that the AR might initially

be down regulated post-exercise, but as recovery continues an up-regulation of the AR is observed. Yet, not all studies have shown similar results. Kvorning *et al.* (2007) has shown no significant change in AR mRNA expression 4 and 24 hours after a strength training session in young men. Similar results have been shown in young (25-30 year) and older (60-65 year) men with no significant difference in AR mRNA expression or protein concentration 1 and 48 hours after heavy resistance exercise bout (J. P. Ahtiainen et al., 2011). However, Ahtiainen *et al.* noted that significant individual differences were observed within the groups. The AR mRNA and protein expression appears to show a phasic response in some, but not all studies. Circulating testosterone and/or resistance exercise has shown to influence AR expression in several studies, yet additional mechanisms regulating AR expression needs to be elucidated.

Conclusion

Testosterone is the primary androgenic sex steroid hormones which effects are ubiquitous. Production is primary synthesized within the testes in adult males, which is tightly regulated through the HPG axis. Testosterone being lipid based requires transport proteins (SHBG and albumin) to be transported within the blood. However, a small percentage (~2-3%) of total testosterone is considered free testosterone and is the most biologically active (Hammond, et al., 1980; Rommerts, et al., 2004). This biologically active testosterone mediates its effects through binding to a ligand activated nuclear hormone AR. The AR, once bound by testosterone, will translocate into the nuclease of the cell. Within the nucleus of the cell the testosterone/AR complex will bind to a specific region of the DNA known as the ARE. Thus, controlling for changes in transcription and/or translation rates of specific genes (Kicman, 2010). Testosterone also has the

ability to alter the expression of IGF-1 and myostatin, which are key regulators of skeletal muscle. Specifically, the IGF-1 gene has shown to contain an ARE which indicates testosterone has the ability to up-regulate IGF-1 expression (Wu, et al., 2007). Myostatin also contains an ARE which, when activated through androgen binding, decreases myostatin expression (Ma, et al., 2001). Thus, testosterone has the ability to directly control the rate of skeletal muscle adaptation by controlling the rate of transcription and/or translation to make new proteins, but also controlling the activity of IGF-1 and myostatin.

Both acute bouts of resistance exercise and resistance training have shown to promote skeletal muscle adaptation by increasing muscle protein synthesis (Abernethy, et al., 1994; Fitts & Widrick, 1996). Specific exercise protocols which emphasis moderate-to-high metabolic demand have shown to increase both testosterone and IGF-1 levels systemically (W. J. Kraemer, et al., 1990). Acute resistance exercise has also shown to reduce myostatin expression (Drummond, et al., 2009; Louis, et al., 2007). In addition to the hormonal response to resistance exercise, the androgen receptor itself has shown to change expression. The AR has shown a phasic response with an initial down-regulation post-exercise followed by an up-regulation as recovery continues (Hulmi, Ahtiainen, et al., 2008; Vingren, et al., 2010).

The ability of an acute increase in endogenous serum testosterone resulting from resistance exercise to increase skeletal muscle testosterone, DHT, and androgen receptor expression still needs to be elucidated. In addition, clarifying the role of the increases in systemic testosterone to alter IGF-1 and myostatin expression, both systemically and locally, is also warranted.

CHAPTER THREE

Methods

Participants

Ten apparently healthy, resistance-trained [regular, consistent resistance training (i.e. thrice weekly) for at least 1 year prior to the onset of the study], men between the ages of 18-30 volunteered to serve as participants in this study. Enrollment was open to men of all ethnicities. Only participants considered as low risk for cardiovascular disease and with no contraindications to exercise as outlined by the American College of Sports Medicine (ACSM), and who had not consumed any nutritional supplements (excluding multi-vitamins) three months prior to the study were allowed to participate. All eligible subjects signed university-approved informed consent documents and approval was granted by the Institutional Review Board for Human Subjects. Additionally, all experimental procedures involved in the study conformed to the ethical consideration of the Helsinki Code.

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Study Site

In the Department of Health, Human Performance, and Recreation at Baylor University, all familiarization and testing sessions were performed within the Baylor Laboratories for Exercise Science & Technology (BLEST). All sample analyses were completed in the Exercise and Biochemical Nutrition Laboratory (EBNL) at Baylor University.

Study Design

Table 1 and Figure 1 provide an outline of the study. In a randomized, cross-over design, participants visited the laboratory on 5 separate occasions in the following manner: visit 1 = entry/familiarization session, visit 2 = testing/resistance exercise session 1, visit 3 = 24 hour follow-up for session 1, visit 4 = testing/resistance exercise session 2, visit 5 = 24 hour follow-up for session 2. Relative to the testing sessions (visits 2 & 4), participants performed a resistance exercise session involving the knee extension exercise on two occasions separated by one week. One session constituted a lower-volume, control session (LV) and was preceded by rest and the other was a higher-volume, experimental session (HV) and proceeded by a bout of moderate-intensity, upper-body resistance exercise using short rest periods (Spiering, et al., 2009).

Independent and Dependent Variables

The independent variable was the resistance exercise protocol [control, lower-volume (LV) vs. experimental, higher-volume (HV)]. Dependent variables in serum included: free and total testosterone, and IGF-1. In skeletal muscle, the variables included: testosterone, DHT, androgen receptor mRNA expression, androgen receptor protein expression, muscle-specific IGF-1 and myostatin mRNA expression.

Entry and Familiarization Session

Participants were recruited through online advertisement and flyers placed within the surrounding Waco, TX area. Participants expressing interest in participating in the study were interviewed on the phone or via email to determine whether they appeared to qualify to participate in this study. Participants believed to meet the eligibility

requirement for training status [resistance trained (i.e. thrice weekly) for at least 1 year prior to the onset of the study] where then invited to attend an entry/familiarization session. Once reporting to the lab, participants completed a medical history questionnaire and underwent a general physical examination to determine whether they met the remaining eligibility criteria. Participants meeting entry criteria were familiarized to the study protocol via a verbal and written explanation outlining the study design, read and signed informed consent documents, and then underwent assessments for body composition and muscle strength. At the conclusion of the familiarization session, participants were given an appointment in which to attend their first testing session. In addition, each participant was instructed to refrain from exercise for 48 hours, fast for 8-hours, and record their dietary intake for four days prior to each of the two testing sessions involved in the study.

Anthropometric and Body Composition Testing

Total body mass (kg) was determined by using a calibrated electronic scale with a precision of ± 0.02 kg (Detecto, Webb City, MO). Total body water (total, intracellular, and extracellular) was determined through use of Bio-Impedance Spectrum Analyzer (BIS) (Xitron 4200, ImpediMed Ltd, Australia). The subjects lied in a supine position for four minutes on a table and were swabbed with an alcohol pad on their right hand and foot. Four electrodes were placed on the body at least five cm apart to allow a low energy variable current between 50μA and 700μA scanning 50 frequency ranging from 5 kHz to 1000 kHz to flow through the body. (BIS) measured the resistance to the current within the body. One current- injection (negative) and one voltage- detector (positive) electrodes were placed on the right foot and hand. One positive electrode was placed on

the posterior surface of the right wrist, mid-line between the radial and ulna styloid processes. The other negative electrode was placed on the posterior surface of the right hand and the distal base of the second metacarpal, ≥ 5 cm apart. The negative electrodes were placed on the foot. One positive electrode was placed on the anterior surface of the right foot with the other negative electrode placed at the distal end of the first metatarsal, ≥ five cm apart. Once connected, the participant's age, gender, weight, and height nearest 0.1cm/kg or inch/lb was entered into the unit and the analysis was started.

Percent body fat, fat mass, fat-free mass, and bone mineral content were determined using dual-energy x-ray absorptiometer (DEXA) [Hologic Discovery, Bedford, MA]. The participants lied in a supine position in only shorts and a t-shirt. The participants were asked to lie motionless for approximately six minutes while the scan was being performed. The subjects were exposed to a low dosage of radiation at each scan. Approximately 1.5 mR of radiation was emitted during the scan. The maximal amount of x-ray radiation exposure per year for non-occupation exposure is 500 mR; the radiation exposure was not significantly more than the background radiation in the local Waco area. Once the scan was completed, it was analyzed following completion of the entry session. The DEXA scans were segmented into regions (right & left arm, right & left leg, and trunk). Each of these segments was analyzed for fat mass, lean mass, and bone mineral content.

Muscle Strength Assessments

In order to determine muscular strength, participants performed one-repetition maximum (1-RM) tests on the bench press, overhead shoulder press (Nebula, Versailles, OH), seated row, and knee extension (Cybex, Medway, MA) exercises while attending

the familiarization session. Participants warmed up by completing 5 to 10 repetitions at approximately 50% of the estimated 1-RM. The participants rested for one minute, and then completed three to five repetitions at approximately 70% of the estimated 1-RM. The weight was then increased conservatively, and the participant attempted to lift the weight for one repetition. If the lift was successful, the participant rested for 2 minutes before attempting the next weight increment. This procedure continued until the participant failed to complete the lift. The 1-RM was then recorded as the maximum weight that the participant was able to lift for one repetition. Test-retest reliability of performing these strength assessments on subjects within our laboratory has demonstrated low mean coefficients of variation and high reliability for the bench press (1.9%, intraclass r = 0.94).

Heart Rate and Blood Pressure

At visits 1-5, heart rate and blood pressure were assessed. At the entry and familiarization session, these variables were obtained as part of the health history assessment. At visits 2 and 4, heart rate and blood pressure was obtained at each of the seven time points in which blood samples were obtained. Heart rate and blood pressure was also obtained at visits 3 and 5. Heart rate was determined by palpation of the radial artery using standard procedures. Blood pressure was assessed in the supine position after resting for five minutes with a mercurial sphygmomanometer using standard procedures.

Dietary Analysis

Participants were required to record their dietary intake for four days (three weekdays and one weekend) prior to each of the two resistance exercise sessions. The participants' diets were not standardized and participants were asked not to change their dietary habits during the course of the study. The dietary recalls were evaluated with the Food Processor Dietary Assessment software program (EHSA Research, Salem, OR) to determine the average daily Kcal and macronutrient consumption of fat, carbohydrate, and protein in the diet for the duration of the study.

Resistance Exercise Protocol

During the control, lower-volume (LV) session, participants performed five sets of 5-RM (90%-95% 1-RM) of the bilateral knee extension exercise with three minutes of rest between sets. However, during the experimental, higher-volume (HV) session, participants performed in the following order, an upper-body resistance exercise protocol of four sets of 10-RM each of the bench press, seated row, and overhead shoulder press exercises immediately prior to the knee extension protocol. For the upper-body protocol, the initial load was set at 80% 1-RM for each participant. If muscle fatigue/failure occurred during a set, a spotter provided assistance until the participant completed the remaining repetitions and resistance was reduced for subsequent sets. In all cases, two minutes of rest separated sets and exercises. Within two minutes, participants began the knee extension exercise protocol identically as performed during the LV session. All training sessions where conducted in the Baylor Laboratories for Exercise Science & Technology (BLEST) and supervised by study personnel.

Hydration Status

Prior to both LV and HV resistance exercise sessions, hydration status was assessed through a urine sample provided immediately prior to each testing sessions. The urine samples were measured for urine specific gravity (USG) determined by a Clinitek Status+ Analyzer (Siemens, Tarrytown, NY). Urine specific gravity is the relative density of urine versus water which is measured by urine refractometry. This method has been established as an accurate measurement of hydration status in both athletic and non-athletic populations (Armstrong et al., 1994; Oppliger, Magnes, Popowski, & Gisolfi, 2005). Previous research has demonstrated individuals who are hypo-hydrated will have an attenuated testosterone response with resistance exercise (Judelson et al., 2008). Adequate hydration was established if urine specific gravity was ($\leq$ 1.02). If participants were classified as dehydrated (urine specific gravity >1.02) participants ingested water until hydration status was met or the testing session was rescheduled.

Blood Sampling

Venous blood samples were obtained in 10 ml vacutainer tubes from a 20 gauge intravenous catheter inserted into the antecubital vein. Blood samples were allowed to stand at room temperature for 10 minutes and then centrifuged for 15 minutes. The serum was removed and frozen at -80°C for later analysis. Eight blood samples were obtained at each of the two resistance exercise sessions, with a total of 16 blood samples being obtained during the course of the study. At each testing session, blood samples were obtained: immediately prior to the commencing the testing session, immediately prior to lower-body exercise, immediately after lower-body exercise, 0.5 hour after

exercise, 1 hour after exercise, 2 hours after exercise, 3 hours after exercise, and 24 hours after exercise.

Muscle Biopsies

Percutaneous muscle biopsies (50-70 mg) were obtained from the middle portion of the vastus lateralis muscle at the midpoint between the patella and the greater trochanter of the femur at a depth between one and two cm. The skin was topically anesthesized (1.5 ml 1% Lidocaine) prior to the incision. After the initial biopsy, the remaining biopsies were made to extract tissue from approximately the same location as the initial biopsy by using the pre-biopsy scar, depth markings on the needle, and a successive incision that was made approximately 0.5 cm to the former from medial to lateral. After removal, adipose tissue was trimmed from the muscle specimens and was immediately frozen in liquid nitrogen and then stored at -80°C for later analysis. Four muscle samples were obtained at each of the two resistance exercise sessions, with a total of eight muscle samples being obtained during the course of the study. At each testing session, muscle samples were obtained: immediately prior to commencing the testing session, immediately after lower-body exercise, 3 hours after exercise, and 24 hours after exercise.

Blood Serum Analysis

Serum Free and Total Testosterone

From the 16 total blood samples obtained from the LV and HV resistance exercise sessions, total and free testosterone levels was determined using commercially available enzyme-linked immunosorbent assays (ELISA) kits (Alpha Diagnostic International, San

Antonio, TX) with a microplate reader (xMark Microplate Absorbance Spectrophotometer, Bio-Rad, Hercules, CA).

The total testosterone assay is based upon competitive solid phase ELISA. Serum samples compete with enzyme-linked testosterone for a fixed and limited number of antibody binding sites. The specificity of the kit for testosterone is 100% with the sensitivity estimated to be at 0.02 ng/ml. Standards, samples, and controls (50 µl) were pipetted in duplicate into a microtiter plate. Testosterone-horseradish peroxidase (HRP) conjugate (100 µl) was pipetted into each well. The plate was covered and incubated on a plate shaker (200 rpm) at room temperature for sixty minutes. The antibody-bound testosterone remained in the microtiter wells. The unbound testosterone was removed by washing three times with an automated plate washer. 3,3',5,'5-Tetramethylbenzidine (TMB) substrate (150 µl) was placed into each well and mixed gently for 5-10 seconds. The plate was then covered and incubated on a plate shaker (200 rpm) at room temperature for 15 minutes. A blue color was developed when TMB was mixed with the antibody bound testosterone-HRP conjugate. The reaction was stopped after the incubation by the addition (50 µl) of a stop solution (sulfuric acid) which turned the blue color to yellow. The intensity of the yellow color is inversely proportional to the concentration of the testosterone within the samples. The absorbance was measured within 15 minutes at 450 nm using a microplate reader. All samples were run in duplicate against a known standard curve. Data analysis was performed using Microplate Manager 6 Software (Bio-Rad, Hercules, CA). The concentrations were calculated against the known reference standards using a 4-parameter curve. The curve fit statistics were R^2 = .998, .998, 1, and .998. The intra-assay coefficient of variation (CV %) for

each of the four plates utilized were 4.3, 2.8, 3.6, and 4.0 %, respectively. The mean inter-assay CV was calculated at 8.7%.

The free testosterone assay is based upon competitive binding ELISA of human serum free testosterone (testosterone unbound to sex hormone binding globulin and albumin). The specificity of the kit for free testosterone is 100% with the sensitivity estimated to be at 0.17 pg/ml. Standards, samples, and controls (25 µl) were pipetted in duplicate into a microtiter plate. Free testosterone-HRP conjugate (100 µl) was pipetted into each well. The plate was then covered and incubated on a plate shaker (575 rpm) at 37^{o} C for 60 minutes. The antibody-bound free testosterone remained in the microtiter wells. The unbound testosterone was then removed by washing three times with an automated plate washer. TMB substrate (150 µl) was placed into each well and mixed gently. The plate was then covered and incubated at 37^{o}C for 15 minutes. After the addition of the TMB a blue color develops. This enzymatic reaction is inversely related to the amount of free testosterone within the samples. The reaction is stopped after the incubation by the addition (50 µl) of a stop solution which turns the blue color to yellow. The absorbance was measured within 15 minutes at 450 nm using a microplate reader. All samples were run in duplicate against a known standard curve. Data analysis was performed using Microplate Manager 6 software (Bio-Rad, Hercules, CA). The concentrations were calculated against the known reference standards using a 4-parameter curve. The curve fit statistics were R^2 = .998, .998, 1 and 1. The intra-assay coefficient of variation (CV %) for each of the four plates utilized were 12.4, 7.5, 6.1, and 3.3%, respectively. The mean inter-assay CV was calculated at 26. 7%.

Skeletal Muscle Analysis

Total RNA Isolation

Approximately 10-15 mg of muscle tissue was used for biochemical analysis. Total cellular RNA was extracted from homogenate of biopsy samples with a monophasic solution of phenol and guanidine isothiocyanate contained within the TRI-reagent (Sigma Chemical Co., St. Louis, MO). The total RNA isolation method was appropriately modified for smaller muscle samples to yield approximately 0.1-9.15 μg/mg muscle tissue. One ml per 50-100 mg of tissue or ~500 μL of TRI-Reagent was added to each tube, and then the muscle samples were homogenized using a pestle. 0.2 ml per ml of TRI Reagent used or ~100 μL of chloroform was added to each tube and shaken, then allowed to sit for 15 minutes. The samples were separated into three distinct phases, a lower (pink) organic phase which contains the protein, a middle (gray) interphase containing the DNA, and an upper (clear) aqueous phase containing the RNA. Using a sterile transfer pipette, the clear aqueous phase was transferred into a new microfuge tube. The remaining interphase and organic phase were stored in an ultra-low freezer at -80^{o}C. Subsequently, 250 μL of 100% isopropanol was then added to each tube and allowed to sit at room temperature for 5-10 minutes. Samples were then centrifuged at 12,000 x g at 2-8^{o}C for 10 minutes, allowing for the formation of a RNA pellet. The supernatant was discarded, then 500 μL of 75% ethanol was added then vortexed to wash the pellet. The samples were centrifuged at 7500 x g at 2-8^{o}C for five minutes, then the supernatant was discarded. The washing proceeded was repeated twice. The pellet was allowed to air dry for 5-10 minutes, then 50 μL of nuclease free water was added. The total RNA concentration was determined spectrophotometerically (SmartSpec Plus, Bio-

Rad, Hercules, CA, USA) by optical density (OD) at 260 nm using an OD_{260} equivalent to 40 μg/μl and the final concentration expressed relative to muscle wet-weight. Test-retest reliability of performing this procedure of total RNA expression on samples in this laboratory has demonstrated low mean coefficients of variation and high reliability (1.8%, intraclass $r = 0.96$). Aliquots of total RNA (5μl) were separated with 1% agarose gel electrophoresis, ethidium bromide stained, and monitored under an ultraviolet light (Chemi-Doc XRS, Bio-Rad, Hercules, CA) to verify RNA integrity and absence of RNA degradation, indicated by prominent 28s and 18s ribosomal RNA bands, as well as an OD_{260}/OD_{280} ratio of approximately 2.0. The RNA samples were stored at -80°C until later analysis.

Reverse Transcription and cDNA Synthesis

Five μg of total skeletal muscle RNA was reverse-transcribed to synthesize cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Each reverse transcription reaction mixture was incubated at 25°C for five minutes, 42°C for 30 minutes, heated to 85°C for 5 minutes, and then quick-chilled on ice. The cDNA concentration was determined by using an OD_{260} equivalent to 50 μg/μl and starting cDNA template concentration was standardized by diluting all samples to 200 ng in nuclease free water prior to amplification.

Oligonucleotide Primers for PCR

The mRNA sequences of human skeletal muscle β-actin (NM_001101), AR (NM_000044), IGF-1 (M37483), MGF (U40870), myostatin (NM_005259), published in the NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov) was used to construct

PCR primers using Beacon Designer software (Bio-Rad, Hercules, CA, USA), and then commercially synthesized (Integrated DNA Technologies, Coralville, IA). These primers amplify respective fragments 150, 140, 145, base pairs (bp) for IGF-1, MGF, and myostatin. Due to its consideration as a constitutively expressed "housekeeping gene," and the fact that it has been shown to be an appropriate external reference standard in human skeletal muscle using real-time PCR in our laboratory, β-actin was used for detecting the relative change in the quantity of mRNA in response to resistance exercise. For β-actin, these primers amplify a PCR fragment of 135 bp.

Real-Time PCR Amplification and Quantitation

Two hundred ng of cDNA template was added to iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and each PCR reaction was amplified using real-time quantitative PCR (iCycler IQ Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA). The amplification profile was run for 40 cycles employing a denaturation step at 95°C for 30 seconds, primer annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. Fluorescence was measured after each cycle resulting from the incorporation of SYBR green dye into each amplicon. The expression of mRNA was determined from the ratio of the threshold cycle (C_T) values relative to β-actin. The specificity of the PCR was demonstrated with an absolute negative control reaction containing no cDNA template, and a single gene product was be confirmed using DNA melt curve analysis. Positive amplification of the amplicons was assessed with agarose gel electrophoresis illuminated with UV transillumination (Chemi-Doc XRS, Bio-Rad, Hercules, CA, USA).

Total DNA Isolation

Using the remaining homogenate from the RNA isolation procedure, DNA was precipitated from the interphase and organic phase by adding 0.3ml of 100% ethanol per 1 ml of TRI Reagent used in the sample preparation or ~ 150 μL. The solution was mixed and allowed to stand for 2-3 minutes at room temperature before centrifuging at 2,000 x g for five minutes at 2-8^{o}C. The supernatant was removed and transferred to a new microfuge tube and saved for total protein isolation. The DNA pellet was then washed for 30 minutes in 0.1 M trisodium citrate and 10% ethanol solution. 1 ml of wash solution was used for every 1 ml of TRI reagent or ~ 500 μL. The DNA pellet was centrifuged at 2,000 x g for five minutes at 2-8^{o}C, repeated twice. The DNA pellet was resuspended in 75% ethanol, 1.5-2 ml for each ml of TRI Reagent or ~ 750 μL. The pellet was allowed to sit at room temperature for 10-20 minutes, and then the ethanol was removed. The DNA pellet was air dried for 5-10 minutes and then dissolved in 150 μL of 8 mM NaOH (sodium hydroxide) with repeated low pipetting. The supernatant was then centrifuged at 12,000 x g for ten minutes to remove any insoluble material then transferred to a new tube. The total DNA concentration was determined spectrophotometerically (SmartSpec Plus, Bio-Rad, Hercules, CA, USA) by optical density (OD) at 260 nm using an OD_{260} equivalent to 50 μg/μl and the final concentration was expressed relative to muscle wet-weight. The DNA was stored in nuclease free water at -80^{o}c for later analysis. Test-retest reliability of performing this procedure of DNA expression on samples within this laboratory has demonstrated low mean coefficients of variation and high reliability (1.6%, intraclass r = 0.95).

Skeletal Muscle Androgen Receptor mRNA Expression

From the eight muscle tissue samples obtained at the LV and HV resistance exercise sessions, the mRNA expression of the androgen receptor gene was performed using real-time PCR based on our previously established laboratory guidelines. Oligonucleotide primers were designed using Primer Express from known human mRNA sequences available online through the NCBI database. A reaction mix (22 µl) consisting of SYBR green super mix, forward and reverse primers, and nuclease free water was combined with (3 µl) of cDNA template. The quantity of mRNA was determined relative to the expression of β-actin, and ΔC_T values were used to compare gene expression. The specificity of the PCR was demonstrated with an absolute negative control reaction containing no cDNA template, and single gene products confirmed using DNA melt curve analysis.

Skeletal Muscle IGF mRNA

From the eight muscle tissue samples obtained at the LV and HV resistance exercise sessions, the mRNA expression of the skeletal muscle IGF gene was performed using real-time PCR based on our previously established laboratory guidelines. Oligonucleotide primers were designed using Primer Express from known human mRNA sequences available online through the NCBI database. A reaction mix (22 µl) consisting of SYBR green super mix, forward and reverse primers, and nuclease free water was combined with (3 µl) of cDNA template. The quantity of mRNA was determined relative to the expression of β-actin, and ΔC_T values were used to compare gene expression. The specificity of the PCR was demonstrated with an absolute negative control reaction

containing no cDNA template, and single gene products confirmed using DNA melt curve analysis.

Skeletal Muscle Myostatin mRNA

From the eight muscle tissue samples obtained at the LV and HV resistance exercise sessions, the mRNA expression of the skeletal muscle IGF gene was performed using real-time PCR based on our previously established laboratory guidelines. Oligonucleotide primers were designed using Primer Express from known human mRNA sequences available online through the NCBI database. A reaction mix (22 µl) consisting of SYBR green super mix, forward and reverse primers, and nuclease free water was combined with (3 µl) of cDNA template. The quantity of mRNA was determined relative to the expression of β-actin, and ΔC_T values were used to compare gene expression. The specificity of the PCR was demonstrated with an absolute negative control reaction containing no cDNA template, and single gene products confirmed using DNA melt curve analysis.

Total Muscle Protein Isolation

The remaining organic phase from the RNA isolation was isolated for total protein content (Willoughby et al., 2007). Specifically, 750 µL of isopropanol was added and allowed to sit at room temperature for 10 minutes. The solution was centrifuged at 12,000 x g for 10 minutes at 2-8^{o}C. The resulting supernatant was discarded, and the outstanding pellet was washed in 1 mL of 0.3 M guanidine/95% ethanol, then the pellet was allowed to stand for 20 minutes at room temperature and centrifuged for five minutes at 7,500 x g at 2-8^{o}C. This process was repeated three times. After the wash step, the

supernatant was again discarded, followed by the addition of 1 mL 100% ethanol, vortexed, and allowed to stand at room temperature for 20 minutes. The mixture was then centrifuged for 5-minutes at 7,500 x g at 2-8^{o}C. The supernatant was removed and the pellet was air-dried for 10 minutes. One mL of 1% SDS was added to dissolve the pellet aided by grinding with a plastic pestle. The supernatant was centrifugation for 10 minutes at 10,000 x g at 2-8^{o}C. The supernatant was transferred to new a microfuge tube and stored at -20^{o}C.

Total Protein Concentration

Total protein concentration was measured by DC Protein Assay (Bio-Rad Hercules, CA, USA). The protein assay is a colorimetric assay for protein concentration following detergent solubilization. The assay is based upon the Lowry assay (Lowry, Rosebrough, Farr, & Randall, 1951) in which the reaction reaches 90% of the maximal color development within 15 minutes. The reaction of protein with an alkaline copper tartrate solution (Reagent A) and a Folin reagent (Reagent B) produces a blue color. The color development is primarily due to the amino acids tyrosine and tryptophan, in addition to cystine, cysteine, and histidine. The microplate assay protocol was utilized in order determine the protein concentration for skeletal muscle testosterone, DHT, and androgen protein. DC protein reagent S (20 µl) was added to each ml of reagent A making working reagent A. Protein standards curve were developed ranging from 100 µg to 1600 µg with the same buffer utilized in the sample preparation for testosterone, DHT, and androgen protein ELISA's. Standards and samples (5 µl) were pipetted into a dry microtiter plate. Reagent A (25 µl) was added into each well, followed by reagent B (200 µl). The plate was allowed to mix for five seconds. Any bubbles that formed were

removed with a clean, dry pipet tip. After 15 minutes, the absorbances were read at 750 nm.

Skeletal Muscle Testosterone and DHT

From the eight muscle samples obtained at the LV and HV resistance exercise sessions, testosterone and DHT levels was determined using commercially available enzyme-linked immunosorbent assays (ELISA) kits (Diagnostic Systems Laboratories, Webster, TX) with a microplate reader (xMark Microplate Absorbance Spectrophotometer, Bio-Rad, Hercules, CA).

The testosterone assay is based upon competitive solid phase ELISA. Muscle homogenate supernatant sample concentration competes with enzyme-linked testosterone for a fixed number of antibody-binding sites. The specificity of the kit for testosterone is 100% with the sensitivity estimated to be at 0.02 ng/ml. Standards, samples, and controls (50 µl) were diluted (1:100) in EIA buffer and pipetted in duplicate in a microtiter plate. Testosterone-HRP conjugate (100 µl) were pipetted into each well. The plate was covered and incubated on a plate shaker (200 rpm) at room temperature for 60 minutes. The antibody bound testosterone remains in the microtiter wells. The unbound testosterone was removed by washing three times with an automated plate washer. TMB substrate (150 µl) was placed into each well and mixed gently for 5-10 seconds. The plate was covered and incubated on a plate shaker (200 rpm) at room temperature for 15 minutes. A blue color was developed when the TMB was mixed with the antibody-bound testosterone-HRP conjugate. The reaction was stopped after the incubation by the addition (50 µl) of a stop solution which turned the blue color to yellow. The intensity of the yellow color is inversely proportional to the concentration of the testosterone within

the muscle samples. The absorbance was measured at 450 nm using the microplate reader within 15 minutes. Data analysis was performed using Microplate Manager 6 Software (Bio-Rad, Hercules, CA). The concentrations were calculated against the known reference standards using a 4-paramter curve with the muscled homogenate corrected for total protein concentration determined by the DC protein assay. The curve fit statistics were R^2= .999 and .999. The intra-assay coefficient of variation (CV %) were 3.3 and 1.6%. The inter-assay CV was calculated at 2.5%.

DHT assay is based upon simultaneous binding of DHT from samples and DHT-HRP conjugate to anti-DHT immobilized on the microtiter plate. Free DHT and enzyme-bound DHT compete for a fixed and limited number of antibody binding sites. The specificity of the kit for DHT is 100% with the sensitivity estimated to be 6 pg/ml. Standards, samples, and control (50 µl) were diluted (1:100) in EIA buffer and pipetted in duplicate in a microtiter plate. Diluted DHT-HRP conjugate (100 µl) was pipetted into each well. The plate was covered and incubated on a plate shaker (200 rpm) at room temperature for 60 minutes. The unbound DHT was then removed by washing three times with an automated plate washer. TMB substrate (150 µl) was placed into each well and mixed gently for 5-10 seconds. The plate was covered again and incubated on a plate shaker until a dark blue color developed in standard A (~20 minutes) at room temperature. The blue color is inversely proportional to the amount of DHT present within the sample. The enzymatic reaction was stopped after the incubation by the addition (50 µl) of stop solution which turned the blue color to yellow. The absorbance was measured at 450 nm using the microplate reader within 15 minutes. Data analysis was performed using Microplate Manager 6 Software (Bio-Rad, Hercules, CA). The

concentrations were calculated against the known reference standards using a 4-paramter curve with the muscled homogenate corrected for total protein concentration determined by the DC protein assay. The curve fit statistics were R^2= 1 and 1. The intra-assay coefficient of variation (CV %) were 10.5 and 11.5%. The inter-assay CV was calculated at 20%.

Skeletal Muscle Androgen Receptor Protein Expression

From the eight muscle tissue samples obtained at the LV and HV resistance exercise sessions, activated androgen receptor protein was determined by a nuclear receptor (NR) transcription factor "sandwich androgen receptor (AR) ELISA" (Active Motif, Carlsbad, CA) which utilizes a consensus sequence of the androgen response element located within the promoter of the androgen receptor gene and a specific polyclonal antibody for the androgen receptor (Santa Cruz Biotech, Santa Cruz, CA). The NR Sandwich AR ELISA kit uses two antibodies that each recognizes a distinct epitope of the protein. Muscle samples and controls were diluted (1 µl of nuclear extract in 49 µl diluent buffer). Muscle samples and controls (50 µl) were then pipetted into the microtiter plate. In the blank wells (50 µl) of only the diluent buffer was utilized. All sample and controls were run in duplicate. The plate was then covered and incubated at room temperature with mild agitation (100 rpm) on a rocking platform for one hour. The plate was then washed (3x) in an automated plate washer. The AR antibody (1:200 dilution in diluent buffer) (50 µl) was pipetted into each well. The plate was again covered and incubated at room temperature with mild agitation (100 rpm) on a rocking platform for 60 minutes. The plate was then washed three times in an automated plate washer. The diluted HRP-conjugated antibody (1:1000 dilution in diluent buffer) (50 µl)

was pipetted into each well after the wash. The plate was covered again and allowed to incubate at room temperature with mild agitation (100 rpm) on a rocking platform for one hour. During this time the developing solution was allowed to reach room temperature. The plate was washed again (4x) in an automated plate washer. The developing solution (100 µl) was then pipetted into each well. The plate was covered and allowed to incubate for ten minutes at room temperature protected from direct light. Following the incubation stop solution (100 µl) was added to each well. The absorbances were read at 450 nm wavelength within five minutes of the addition of the stop solution. Data analysis was performed using Microplate Manager 6 Software (Bio-Rad, Hercules, CA). The concentrations were calculated against the known reference standards using liner fit curve with the muscled homogenate corrected for total protein concentration determined by the DC protein assay. The curve fit statistics were $R^2 = .994$. The intra-assay coefficient of variation (CV %) were 3.3 and 4.5%. The inter-assay CV was calculated at 8.3%.

Statistical Analyses

Statistical analyses were performed by utilizing separate 2 x 4 (Session x Test) and 2 x 7 (Session x Test) factorial analyses of variance (ANOVA) with repeated measures. Further analysis of the main effects was performed by separate one-way ANOVAs. Significant between-group differences were then determined involving the Tukey's Post Hoc Test. All statistical procedures were performed using SPSS 20.0 software and a probability level of < 0.05 was adopted throughout (Mills et al., 2009; Putt & Chinchilli, 1999; Saarinen, 2004; Simon & Chinchilli, 2007). The magnitude of the statistical significance was measured by effect size. Partial Eta-Squared was adopted throughout, which estimates the ratio of variance in the dependent variable that is

explained by the independent variable. Partial Eta Squared effects sizes are characterized 0.1 to 0.3 as small, 0.3 to 0.5 as medium, and ≥ 0.5 as large (Bakeman, 2005; Cohen, 1992; Levine & Hullett, 2002). Based on the findings of (Fry & Lohnes, 2010), which determined a resistance exercise induced elevation in serum testosterone [effect size (Cohen's d = 1.27)], a sample size of (N = 9) was calculated for a one-tailed hypothesis to achieve a statistical power of 0.80. To minimize the probability of making a type II error, 10 subjects were recruited for this study.

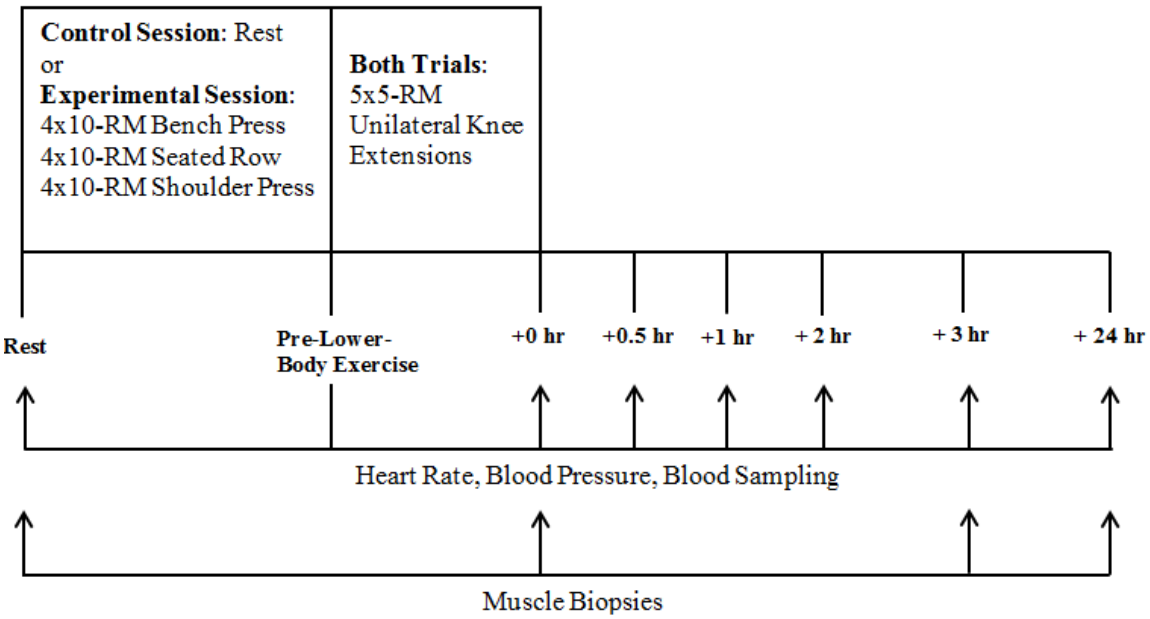


Figure 1. An illustration of the experimental protocol to be used in the study. At each of the 7 time points, heart rate and blood pressure were assessed and blood samples were obtained. Muscle biopsies, however were obtained at rest, +0 hr, +3 hr, and +24hr.

Table 1

Overview of Research Design

Visit 1 (Familiarization and Entry)	Testing Session 1 (Visit 2)	24 Hour Follow-Up (Visit 3)	Testing Session 2 (Visit 4)	24 Hour Follow-Up (Visit 5)
Explanation of Study Procedures	Hydration Status (BIS) & (USG)	Heart Rate and Blood Pressure	Hydration Status (BIS) & (USG)	Heart Rate and Blood Pressure
Demographic and Health History Form Activity Form	Heart Rate and Blood Pressure	Blood Collection	Heart Rate and Blood Pressure	Blood Collection
General Exam To Determine Qualifications to Participate in Study	Blood Collection	Muscle Biopsy	Blood Collection	Muscle Biopsy
Informed Consent Form	Muscle Biopsy		Muscle Biopsy	
Determination of Height and Body Weight	Diet Log Analysis		Diet Log Analysis	
Determination of Resting Heart Rate and Blood Pressure	Resistance Exercise Session		Resistance Exercise Session	
Body Composition Assessment (DEXA)				
Hydration Status (BIS)				
Muscle Strength Assessments				

CHAPTER FOUR

Results

Subject Demographics

Ten participants were initially recruited for the study, completed consent forms, and participated in an initial familiarization session. Of the 10 participants, nine completed the research study, as one participant became ill and was unable to continue. Of the nine who completed the study, eight subjects were utilized in data analysis as one participant's data was considered as a statistical outlier because his levels of free and total testosterone was greater than two standard deviations above the group mean. Table 2 shows the sample size, along with the baseline means (±SD) for height, weight, and age for the eight participants.

Table 2

Participant Baseline Demographics

Control (LV) & Experimental (HV)	
N size	8
Height (cm)	179.07 (5.3)
Weight (kg)	96.69 (15.2)
Age (years)	23.6 (4.8)

Note : Values are reported as means (±SD)

Participant Resistance Training Status

The inclusion criterion for the study was apparently healthy, resistance-trained men. Resistance-trained was defined as [regular, consistent resistance training (i.e. thrice

weekly) for at least 1 year prior to the onset of the study]. For the eight participants utilized for data analysis, resistance training experience was much greater compared to the minimal level required to participate in the study. Based on self-report from each participant, the average resistance training age for all eight participants was 8.57 (±5.09) years.

Testing Time

Endocrine hormone levels are known to fluctuate throughout the day. Specifically, testosterone is known to follow a circadian rhythm, peaking early in the morning roughly 6 am to 10 am (Pardridge, 1986). For each participant, testing time was standardized to occur within this time frame. Table 4 provides the mean (±SD) for the time each morning that participants started each testing session.

Table 3

Morning Testing Times

	Control (LV) & Experimental (HV)
Control	7:24 (1:37)
Experimental	7:06 (1:04)

Note : Values are reported as means (±SD)

Dietary Analysis

Participants recorded their dietary intakes for four days prior to the LV and HV testing sessions. The diet records were used to analyze the average caloric and macronutrient consumption before the testing sessions to measure any possible changes that may have occurred during the course of the testing. Total kcal, protein,

carbohydrates, and fats were grouped together for statistical analysis. Table 5 provides the average values (±SD) for total kcal and macronutrients before each testing session. No significant Group main effect ($p = 0.917$, effect size = 0.000) was seen for total calories. In addition, no significant difference in protein ($p = 0.839$, effect size = 0.003), carbohydrates ($p = 0.542$, effect size = 0.027), or fats ($p = 0.879$, effect size = 0.002) were seen.

Table 4

Dietary Caloric and Macronutrient Intake

	Control (LV)	Experimental (HV)
Total Calories (kcal/day)	3052 (944)	3099 (814)
Protein (g/day)	191 (101)	202 (91)
Carbohydrate (g/day)	313 (76)	332 (40)
Fat (g/day)	113 (43)	110 (34)

Note: Data are represented as means (±SD)

Body Composition

Body composition was measured during the familiarization session for each participant. Analysis of body composition partitioned into a three compartment model consisting of bone mineral content, adipose tissue (i.e. fat mass), and lean mass (i.e. skeletal muscle, organ tissue, body water) which was measured through the use of dual

energy x-ray absorptiometer (DEXA). Table 6 shows the mean (±SD) for each body composition variable.

Table 5

Body Composition

Familiarization	
Bone Mineral Content (Kg)	2.76 (0.41)
Fat Mass (Kg)	17.20 (10.38)
Lean Mass (Kg)	68.20 (5.30)
Body Fat Percentage	18.35 (8.58)

Note: Values are reported as means (±SD)

Hydration Status

Previous research has demonstrated that individuals who are hypo-hydrated will have an attenuated testosterone response with resistance exercise (Judelson, et al., 2008). Due to this concern, hydration status was measured during the familiarization session and before each of the testing sessions. Hydration status can be measured through various methods which include change in body weight, urine specific gravity (USG), and bio-impedance spectroscopy (BIS). BIS specifically accounts for total body water and intracellular and extracellular fluid. Table 7 shows USG, total body weight, and body water (total, intracellular, extracellular) with mean (±SD) for LV, HV, and familiarization sessions (BIS only). No significant Session main effect ($p = 0.246$, effect size = 0.095) was shown for USG between each testing session. No significant Session main effect ($p = 0.95$, effect size = 0.004) was seen in body weight between the two testing sessions. Body water showed no significant difference ($p = 0.966$, effect size = 0.000); ($p = 0.998$,

effect size = 0.003); (p = 0.897, effect size = 0.011) respectively, between the familiarization session and the LV and HV testing sessions.

Table 6

Hydration Status

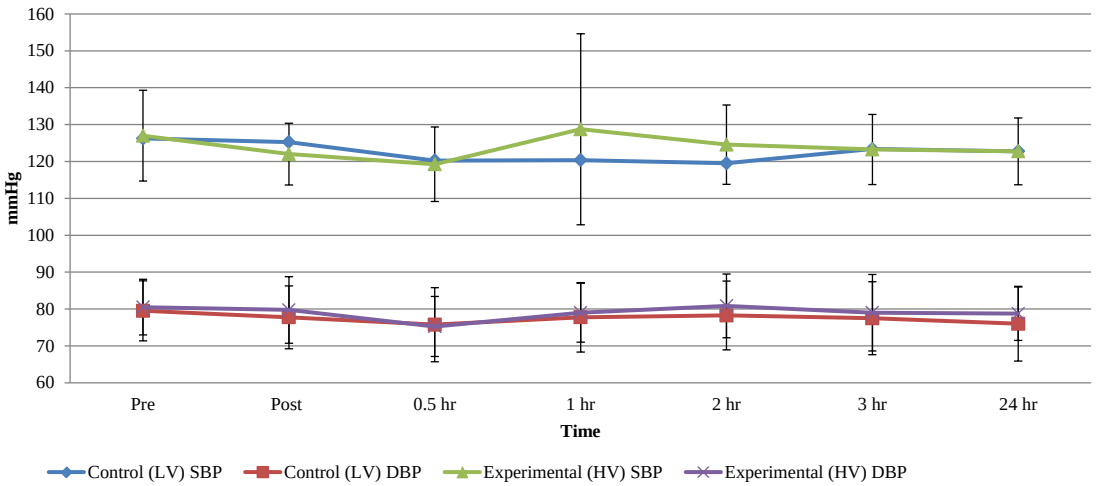
Control (LV) & Experimental (HV)	
Urine Specific Gravity (USG)	
Con: 1.018 (.007)	Exp: 1.021 (.006)
Total Body Weight (Kg)	
Con: 96.94 (15.6)	Exp: 96.44 (15.8)
Body Water (Total, Intra & Extra-Cellular) (Liters)	
TBW - Fam: 52.39 (4.88)	Con: 52.06 (5.34) Exp: 51.72 (4.51)
ICW - Fam: 31.23 (2.48)	Con: 31.31 (2.88) Exp: 31.24 (1.91)
ECW - Fam: 21.16 (2.88)	Con: 20.75 (2.77) Exp: 20.48 (2.88)

Note: Values are reported as means (±SD), Fam: Familiarization session
Con: Control (LV) session, Exp: Experimental (HV) session

Hemodynamic Measurements

Systolic & Diastolic Blood Pressure

Blood pressure (systolic and diastolic) was measured during seven time points for each testing session. No significant Session main effect (p = 0.269, effect size = 0.011) or Time main effect (p = 0.928, effect size = 0.017) was shown for systolic blood pressure. No significant Session main effect (p = 0.150, effect size = .019) or Time main effect (p = 0.87, effect size = 0.023) was shown for diastolic blood pressure (Figure 2).



	Pre	post	30 min	1 hr	2 hr
Control (LV)	126 (15)/80 (8)	125 (17)/78 (9)	120 (8)/ 76 (10)	120(6)/78 (9)	120 (10)/78 (9)
Experimental (HV)	127 (12)/81 (8)	122 (8)/80 (9)	119 (10)/75 (8)	129(26)/79 (8)	125 (11)/81 (9)

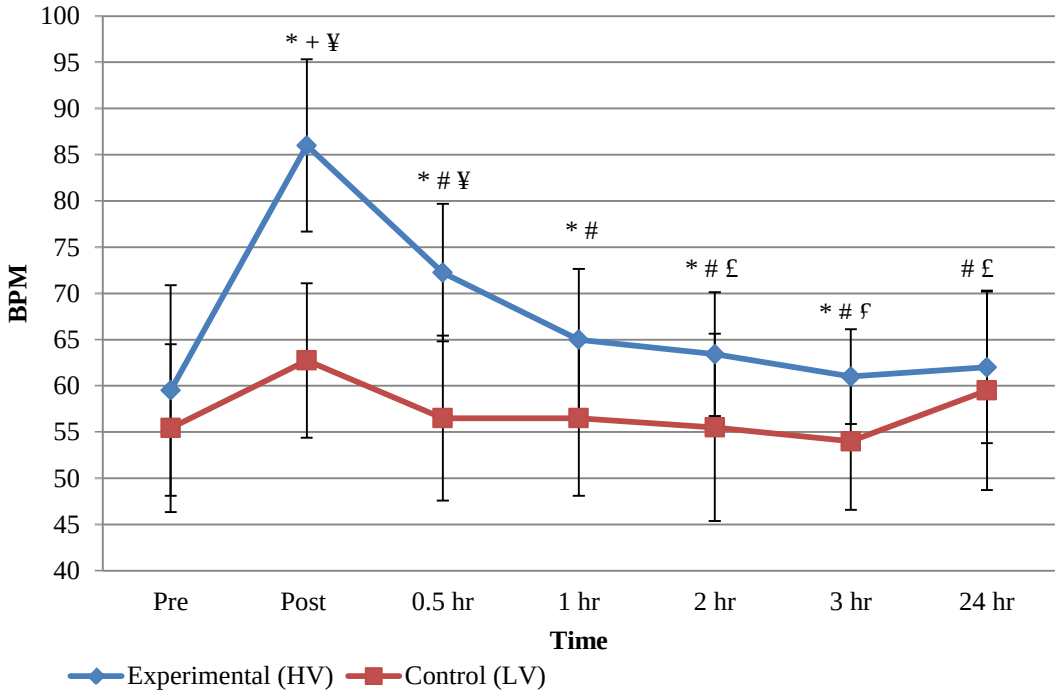
	3 hr	24 hr
Control (LV)	123 (9)/78 (10)	123 (10)/76 (10)
Experimental (HV)	123 (9)/79 (10)	123 (9)/79 (7)

Figure 2. Changes in Systolic / Diastolic Blood Pressure. Data expressed as mean (±SD).

Heart Rate

A significant Session main effect ($p < .0001$, effect size = 0.188) and a significant Time main effect ($p < .0004$, effect size = 0.208) was observed (Figure 3). One way analysis revealed a significant difference ($p < .0001$, effect size = 0.188) between sessions with the experimental (HV) session heart rate being elevated compared to control session. Tukey post-hoc test showed significant elevation post-exercise ($p = 0.0015$), 30 minutes ($p = 0.0149$), 1 hour ($p = 0.0047$), 2 hours ($p = 0.0007$), and 3 hours ($p = 0.0099$). One way analysis revealed no significant Time main effect ($p = 0.602$, effect size = 0.085) for the LV session. The HV session did have a Time main effect ($p < .0001$, effect size = 0.563). Tukey post-hoc test showed significant elevation post-exercise ($p < .0001$), a significant decrease from post-exercise to 30 minutes ($p = 0.0016$), 1 hour ($p < .001$), 2 hours ($p < .001$), 3 hours ($p < .001$), and 24 hours ($p < .001$).

Elevation was also shown pre-exercise to 30 minutes post (p = 0.0032), with a significant decline 30 minutes post to 2 hours (p = 0.434), 30 minutes post to 3 hours (p = 0.0086), and 30 minutes post to 24 hours (p = 0.0161).



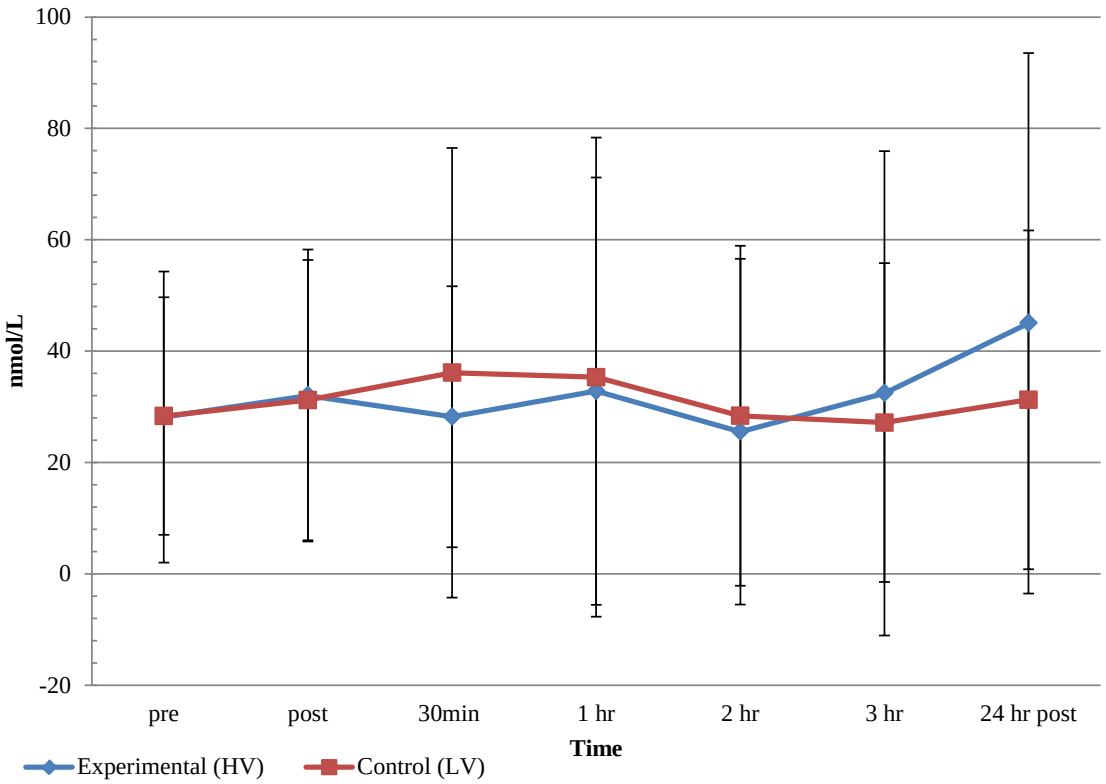
	pre	post	30 min	1 hr	2 hr	3 hr	24 hr post
Control (LV)	55(9)	63(8)	57(9)	57(8)	56(10)	54(7)	60(11)
Experimental (HV)	60(11)	86(9)	72(7)	72(7)	63 (7)	61(5)	62(8)

Figure 3. Changes in Heart Rate. Data expressed as mean (±SD). * indicates a significant increase post (p=.002), 30 min (p=.015), 1 hr (p=.005), 2 hr (p=.001), and 3 hr (p=.001) in Experimental (HV) compared to Control (LV). $^{+}$ increase pre-post (p<.0001) in HV group. $^{\#}$ decrease post-30 min (p=.002), 1 hr (p<.001), 2 hr (p<.001), 24 hr (p<.001) in HV group. $^{¥}$ increase pre- 30 min (p=.003) in HV group. $^{£}$ decrease 30 min – 2 hr (p=.434), 30 min – 3 hr (p=.009), 30 min – 24 hr (p=.016).

Serum Free and Total Testosterone

Serum Total Testosterone

No significant Session main effect (p = 0.108, effect size = 0.026) or Time main effect (p = 0.990, effect size = 0.142) for serum total testosterone was observed (Figure 3).



	pre	post	30 min	1 hr	2 hr
Control (LV)	28.34 (21.33)	31.21 (25.19)	36.13 (40.36)	35.34 (43)	28.4 (30.51)
Experimental (HV)	28.17 (26.13)	32.02 (26.22)	28.21 (23.44)	32.83 (38.36)	25.54 (31.02)

	3 hr	24 hr post
Control (LV)	27.18 (28.61)	31.26 (30.42)
Experimental (HV)	32.43 (43.49)	45.02 (48.53)

Figure 3. Changes in serum Total Testosterone concentrations. Data expressed as mean (±SD).

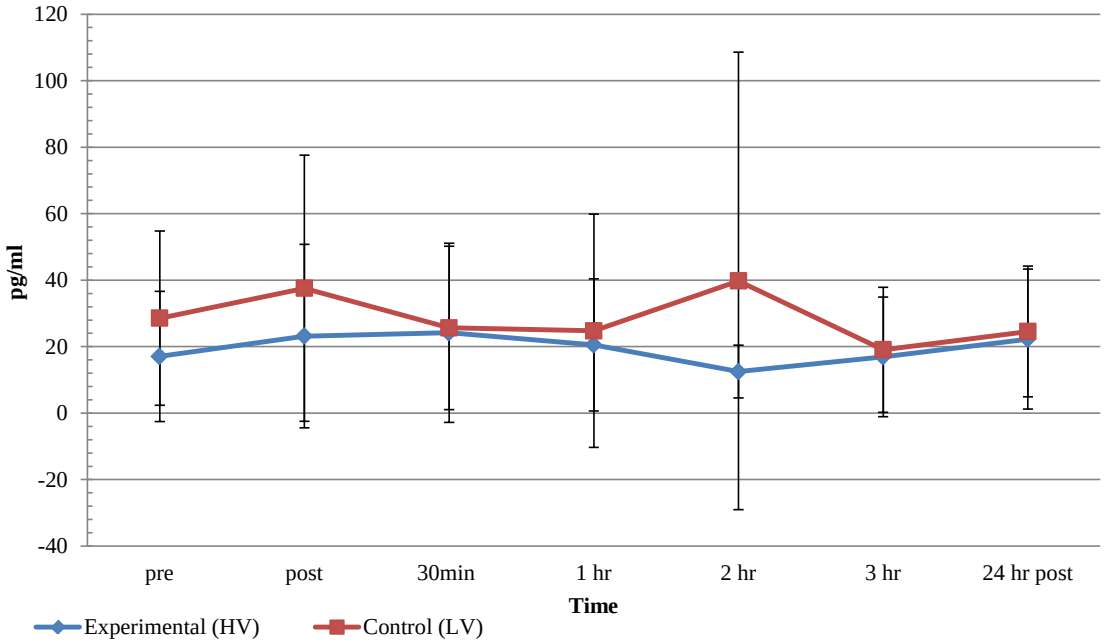
Serum Free Testosterone

No significant Session main effect ($p = 0.066$, effect size = 0.034) or Time main effect ($p = 0.987$, effect size = 0.156) for serum free testosterone was observed (Figure 4). However, a weak statistical trend was observed ($p < 0.10$) for an increase in free testosterone associated with the LV session.

Serum Testosterone Hypothesis Conclusion:

H_1: Following the exercise bout involving high volume (upper- & lower-body) resistance exercise, a significant increase in serum total and free testosterone will occur

compared to the exercise bout involving low volume (lower-body) resistance exercise. Serum total and free testosterone levels did not significantly increase. Therefore, the H_1 hypothesis is rejected.



	pre	post	30 min	1 hr	2 hr
Control (LV)	28.56 (26.23)	37.56 (40.05)	25.64 (24.58)	24.77 (35.08)	39.76 (68.81)
Experimental (HV)	17.06 (19.61)	23.17 (27.6)	24.17 (26.91)	20.53 (19.86)	12.50 (7.93)

	3 hr	24 hr post
Control (LV)	19.05 (18.81)	24.55 (19.68)
Experimental (HV)	16.93 (18.02)	22.27 (21.04)

Figure 4. Changes in serum Free Testosterone concentrations. Data expressed as mean (±SD).

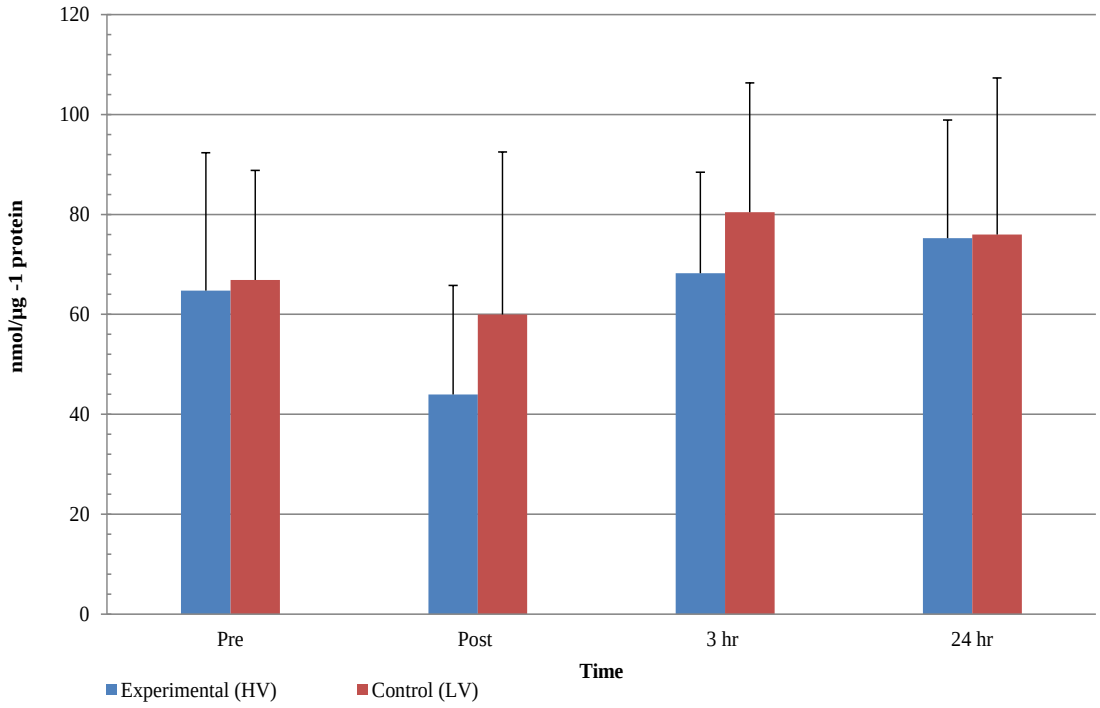
Skeletal Muscle Testosterone & DHT

Muscle Testosterone

No significant Session main effect ($p = 0.082$, effect size = 0.053) or Time main effect ($p = 0.057$, effect size = 2.663) for muscle testosterone concentrations was observed (Figure 5).

Skeletal Muscle Testosterone Hypothesis Conclusion:

H_2: Following the exercise bout involving high volume (upper- & lower-body) resistance exercise, a significant increase in muscle testosterone will occur compared to the exercise bout involving low volume (lower-body) resistance exercise. Skeletal muscle testosterone concentration did not significantly increase. Therefore, the H_2 hypothesis is rejected.



	pre	post	3 hr	24 hr post
Control (LV)	66.88 (21.94)	59.98 (32.52)	80.43 (25.91)	75.98 (31.33)
Experimental (HV)	64.74 (29.48)	43.98 (21.88)	68.21 (21.07)	75.27 (25.25)

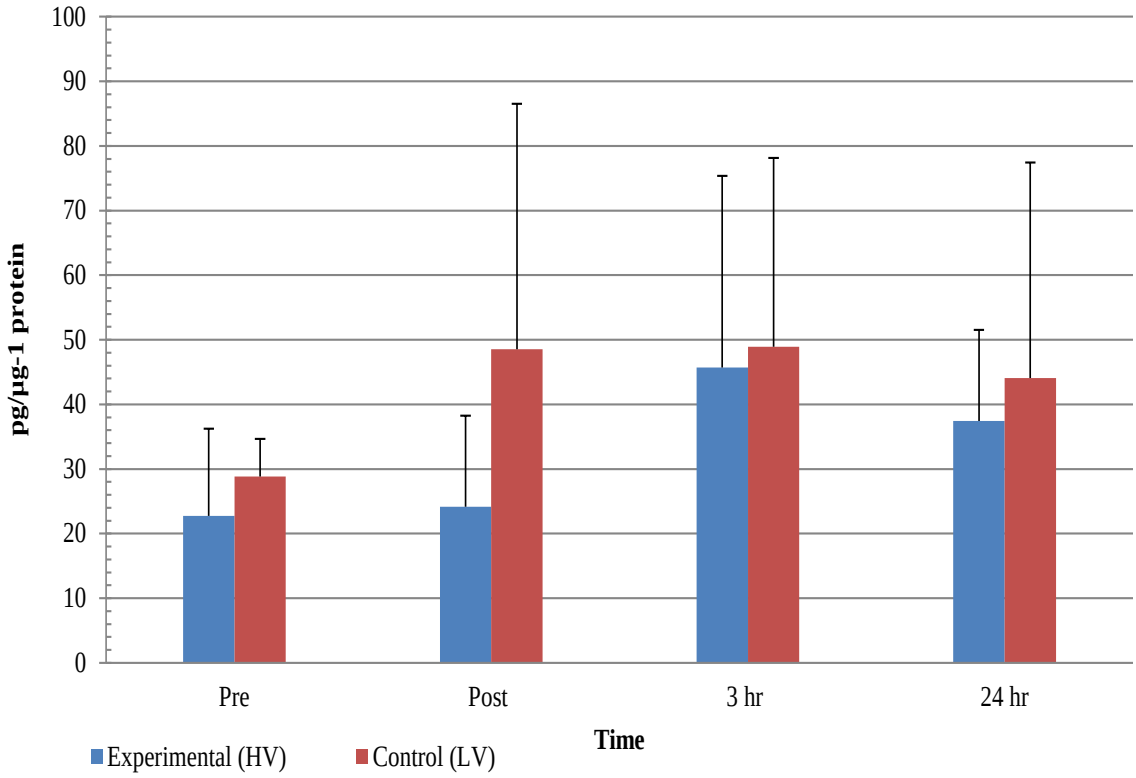
Figure 5. Changes in Muscle Testosterone concentrations per microgram of supernatant protein. Data expressed as mean (±SD).

Muscle DHT

No significant Session main effect ($p = 0.062$, effect size = 0.061) or Time main effect ($p = 0.118$, effect size = 2.045) for muscle DHT concentration was observed (Figure 6).

Skeletal Muscle DHT Hypothesis Conclusion:

H_3: Following the exercise bout involving high volume (upper- & lower-body) resistance exercise, there will be a significant increase in muscle DHT compared to the exercise bout involving low volume (lower-body) resistance exercise. Skeletal muscle DHT concentrations did not significantly increase. Therefore, the H_3 hypothesis is rejected.



	pre	post	3 hr	24 hr post
Control (LV)	28.83 (5.84)	48.55 (37.97)	48.92 (29.24)	44.10 (33.36)
Experimental (HV)	22.77 (13.67)	24.14 (13.87)	45.72 (31.68)	37.43 (14.52)

Figure 6. Changes in Muscle DHT concentrations per microgram of supernatant protein. Data expressed as mean (±SD).

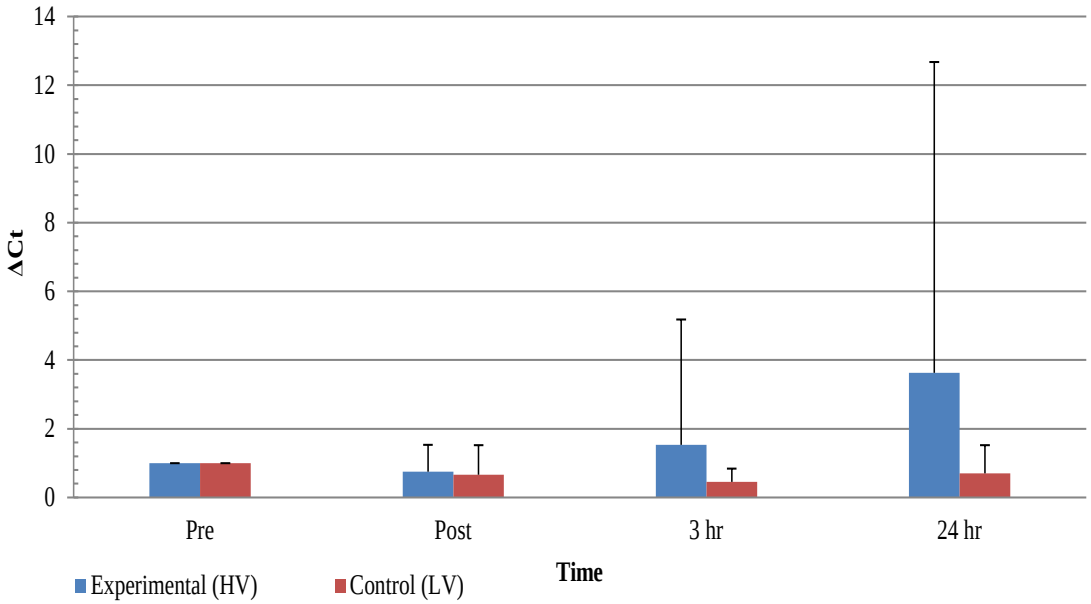
Muscle mRNA Expression

Myostatin mRNA

No significant Session main effect (p = 0.247, effect size = 0.024) or Time main effect (p = 0.648, effect size = 0.553) for muscle myostatin mRNA expression was observed (Figure 7).

Skeletal Muscle Myostatin mRNA Expression Hypothesis Conclusion:

H_4: Following the exercise bout involving high volume (upper- & lower-body) resistance exercise, a significant decrease in muscle myostatin mRNA expression will occur compared to the exercise bout involving low volume (lower-body) resistance exercise. Skeletal muscle myostatin mRNA expression did not significantly decrease. Therefore, the H_4 hypothesis is rejected.



	pre	post	3 hr	24 hr post
Control (LV)	1 (0)	0.662 (0.864)	0.452 (0.385)	0.706 (0.814)
Experimental (HV)	1 (0)	0.747 (0.783)	1.529 (3.653)	3.627 (9.052)

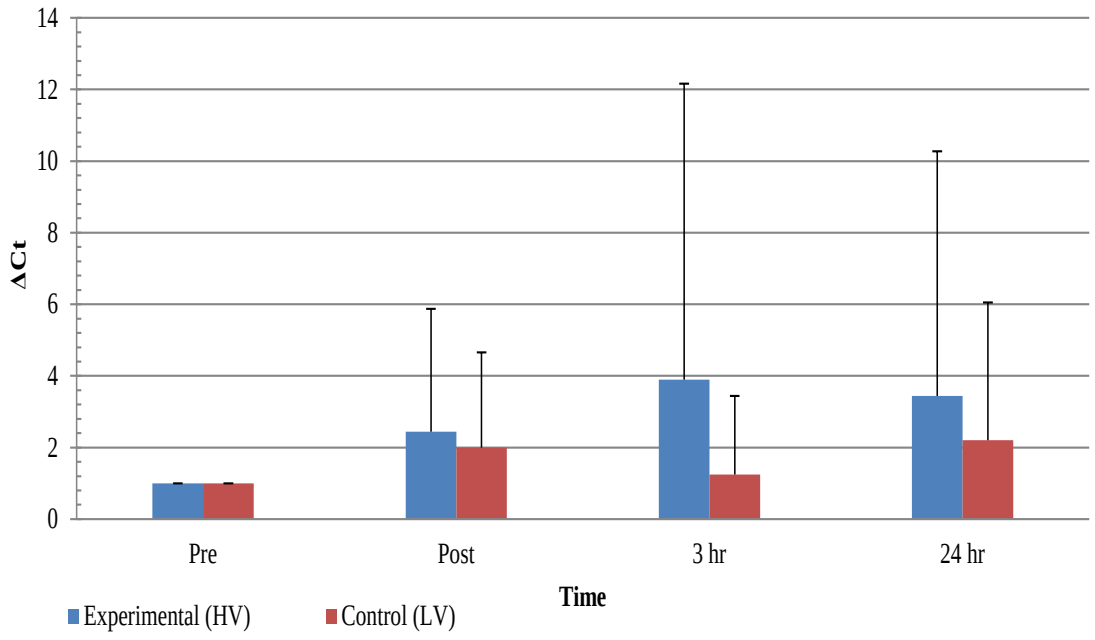
Figure 7. ΔCt in Muscle Myostatin mRNA expression; Data expressed as mean (±SD).

MGF Expression

No significant Session main effect (p = 0.326, effect size = 0.017) or Time main effect (p = 0.654, effect size = 0.545) for muscle MGF mRNA expression was observed (Figure 8).

Skeletal Muscle MGF mRNA Expression Hypothesis Conclusion

H_5: Following the exercise bout involving high volume (upper- & lower-body) resistance exercise, a significant increase in muscle IGF-1 mRNA will occur compared to the exercise bout involving low volume (lower-body) resistance exercise. Skeletal muscle MGF mRNA expression did not significantly increase. Therefore, the H_5 hypothesis is rejected.



	pre	post	3 hr	24 hr post
Control (LV)	1 (0)	1.994 (2.665)	1.243 (2.195)	2.206 (3.841)
Experimental (HV)	1 (0)	2.443 (3.426)	3.9 (8.262)	3.442 (6.833)

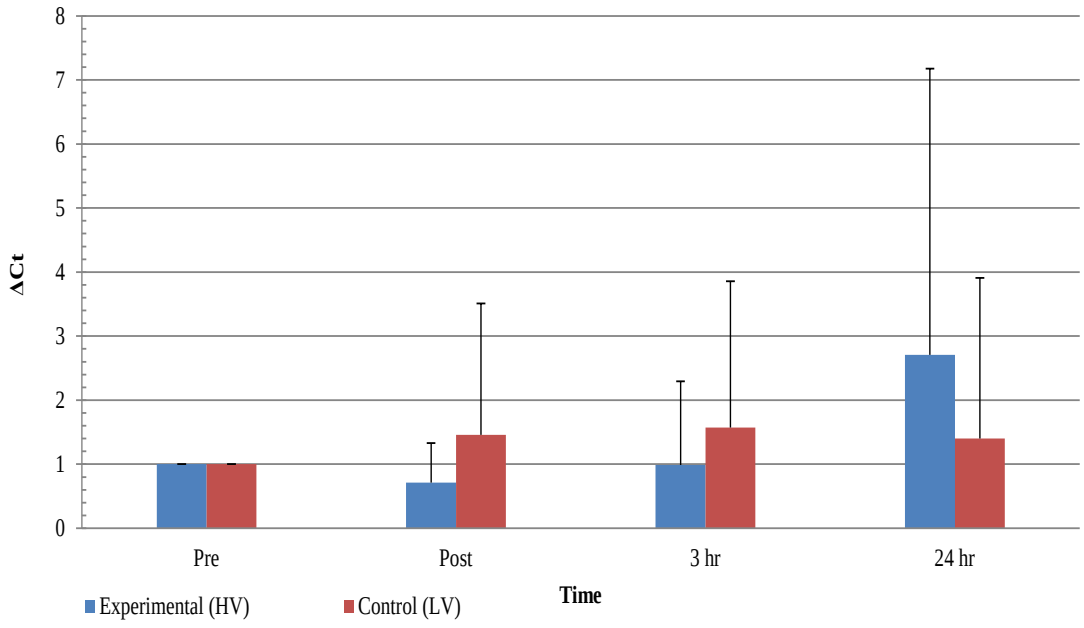
Figure 8. ΔCt in Muscle MGF mRNA expression; Data expressed as mean (±SD).

AR Expression

No significant Session main effect (p = 0.992, effect size = 0.000) or Time main effect (p = 0.507, effect size = 0.786) for muscle androgen receptor mRNA expression was observed (Figure 9).

Skeletal Muscle AR mRNA Expression Hypothesis Conclusion

H_6: Following the exercise bout involving high volume (upper- & lower-body) resistance exercise, there will be a significant increase in androgen receptor mRNA expression will occur compared to the exercise bout involving low volume (lower-body) resistance exercise. No significant increase in skeletal muscle androgen receptor mRNA expression occurred. Therefore, the H_6 hypothesis is rejected.



	pre	post	3 hr	24 hr post
Control (LV)	1 (0)	1.456 (2.055)	1.571 (2.287)	1.402 (2.509)
Experimental (HV)	1 (0)	0.713 (0.619)	0.986 (1.306)	2.709 (4.467)

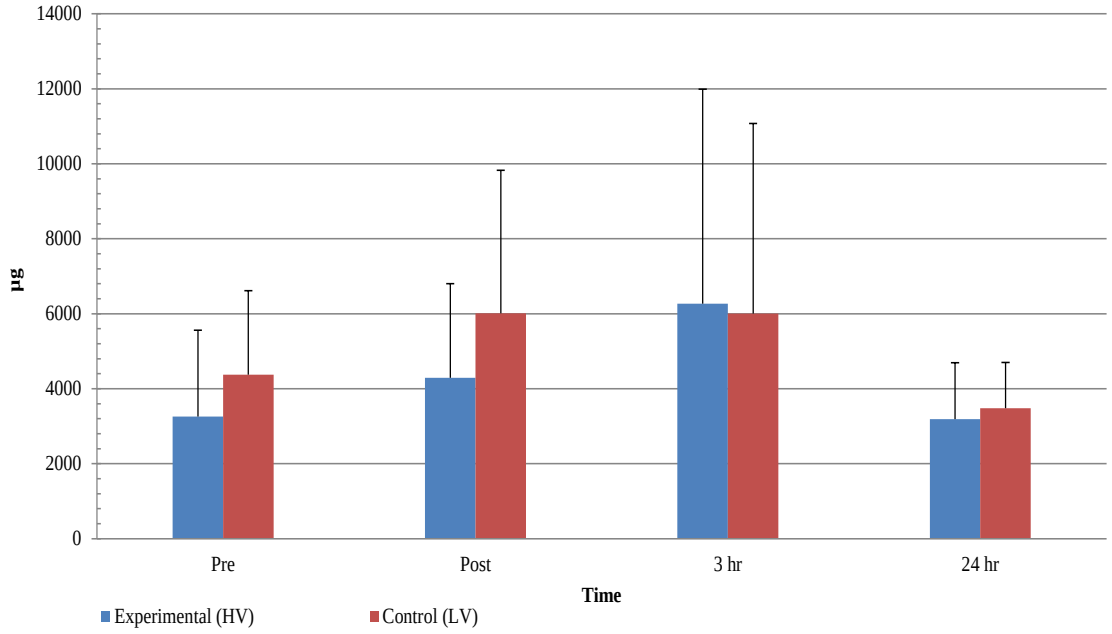
Figure 9. ΔCt in Muscle Androgen Receptor mRNA expression; Data expressed as mean (±SD).

AR Protein Content

No significant Session main effect (p = 0.432, effect size = 0.013) or Time main effect (p = 0.133, effect size = 1.957) for muscle androgen receptor protein content was observed (Figure 10).

Skeletal Muscle AR Protein Content Hypothesis Conclusion

H_7: Following the exercise bout involving high volume (upper- & lower-body) resistance exercise, a significant increase in androgen receptor protein content will occur compared to the exercise bout involving low volume (lower-body) resistance exercise. No significant increase in androgen receptor protein content occurred. Therefore, the H_7 hypothesis is rejected.



	Pre	post	3 hr	24 hr post
Control (LV)	4373.05 (2240.48)	6013.63 (3813.09)	6008.45 (5066.11)	3482.97 (1220.37)
Experimental (HV)	3256.82 (2304.27)	4294.3 (2509.97)	6271.05 (5722.44)	3191.98 (1501.31)

Figure 10. Androgen Receptor protein content; Data expressed as mean (±SD).

CHAPTER FIVE

Discussion

The purpose of this study was to compare the purported increase in serum testosterone levels resulting when a lower-body resistance exercise is combined with upper-body resistance exercise performed immediately before the lower-body resistance exercise. Various studies have demonstrated that resistance exercise will elicit an endogenous testosterone response (W. J. Kraemer, et al., 1991; W. J. Kraemer, Hakkinen, et al., 1999; W. J. Kraemer, et al., 1990; Roberts, et al., 2009; Spiering, et al., 2008; Spiering, et al., 2009). While each study utilized different protocols and various populations, a general consensus on the type of resistance exercise protocol to elicit a testosterone response has been recognized. As such, an exercise bout consisting of high-intensity (load), moderate-to-high volume (set x number of reps x intensity), large muscle groups, and short rest periods will result in an increase in serum testosterone levels. The exact protocol utilized in the present study has previously shown to elicit this type of testosterone response in non-resistance trained participants (Spiering, et al., 2009). Due to this consensus, the primary purpose of this study was to determine if this type of resistance exercise would result in elevations in endogenous testosterone in resistance-trained participants, and if so, would this increased androgenic response result subsequently increase muscle testosterone, DHT, and androgen receptor mRNA expression and protein content. Additionally, if an increase in muscle testosterone levels due to the exercise bout was observed, other key regulators of skeletal muscle [myostatin

and muscle specific IGF-1 peptide (MGF)] were analyzed to determine the androgen-responsiveness in the expression of these two genes.

Serum Free and Total Testosterone

Contrary to the numerous other studies which have shown an elevation in endogenous testosterone levels as a result of resistance exercise, in the present study endogenous total (Figure 3) and free testosterone (Figure 4) did not significantly increase. This is of specific interest due to the fact that the exercise protocol utilized was the same protocol employed by Spiering *et al.* (2009). Spiering *et al.* demonstrated a significant increase in serum testosterone immediately post-exercise within the upper-lower body session, but in non-resistance trained participants. They also showed a significant increase immediately post-exercise and one hour post-exercise compared to the group which only performed the lower-body protocol. Both the study by Spiering *et al.* (2009) and the present study utilized young men (26 ±4 and 24 ±5 years, respectively). However, one key difference between this study and Spiering's study was the resistance training status of the participants. Spiering utilized participants who were not resistance-trained, which was defined as no previous involvement in resistance exercise within six months of the study. However, the present study utilized a resistance-trained population. This was defined as regular, consistent resistance training (thrice weekly) for at least one year prior to the onset of the study. Our participants had extensive experience with an average of 8.6 years resistance training (Table 3). This may have accounted for the difference seen between the two studies and other studies which have employed similar protocols. Cadore *et al.* (2008) demonstrated different hormonal responses in trained (>3 years) versus untrained men (40 ±4 years) after a strength training program. The strength

trained group demonstrated a lower anabolic response compared to the untrained group wherein the untrained group had significant elevations in total and free testosterone after multiple sets of a superset strength training protocol (Cadore et al., 2008). Smilios *et al.* (2003) examined the hormonal response to three types of training protocols (maximum strength, muscle hypertrophy, strength endurance) with 2, 4, and 6 sets. The participants had (2-8 years) resistance training experience. The protocol utilized a combination of upper- and lower-body exercises (bench press, lateral pull downs, squat, overhead press). Testosterone levels were measured pre, post, 15 minutes, and 30 minutes after the resistance exercise session. The maximal strength protocol did not show a significant elevation in testosterone between the sets or at any time point during the session. However, when the same group performed the muscle hypertrophy and strength endurance protocol an elevation in testosterone was observed compared to baseline. This increase was only significant during the 4 sets protocol, not the 2 or 6 sets protocol (Smilios, Pilianidis, Karamouzis, & Tokmakidis, 2003). Limited research has been conducted on what is classified as "forced repetitions". This is when during a resistance exercise set, the individual fails during the concentric portion of the lift. They are then assisted by a training partner during the concentric phase of the movement to help complete the set (S. F. a. W. Kraemer, 2004). Ahtiainen *et al.* (2002) examined the acute hormonal response during forced versus maximum repetition, multiple exercise protocol. The participants were classified as recreationally resistance trained. Serum testosterone levels were measured 15 minutes and 30 minutes post-exercise. Each group performed a lower body protocol consisting of leg press, squat, and knee extensions. No significant increase post-exercise (15 & 30 minutes) was seen in either group. However, a significant

increase in serum cortisol levels was observed at both 15 minutes and 30 minutes post-exercise compared to baseline levels. In the forced repetition group, a significant elevation was seen compared to the maximum repetition group at both the 15 minute and 30 minute time points (J. P. Ahtiainen, Pakarinen, Kraemer, & Hakkinen, 2003). This indicates that the higher total work performed by the forced repetition group did not result in a higher testosterone response. Yet, the forced repetition group resulted in a significant elevation in cortisol.

Hydration Status

Hydration status was accounted for through the use of multiple methods. Research has shown that hypo-hydration can reduce the testosterone response to resistance exercise (Judelson, et al., 2008). To reduce the likelihood of the participants being dehydrated before testing, urine samples were taken before each testing session. Urine specific gravity was utilized to establish hydration (USG $\leq$ 1.020). This method has been shown to be a reliable and accurate method to determine hydration status in both athletic and non-athletic populations (Armstrong, et al., 1994; Oppliger, et al., 2005). However, no single method has been shown to be the "Gold Standard" for determining hydration status (Armstrong, 2007). It has been shown that an acute (500-mL) bolus of water may alter interpretation of hydration status (Sollanek, Kenefick, Cheuvront, & Axtell, 2011). To minimize the possibility of this, hydration status was also measured during the familiarization session and before each testing session by bioelectrical impedance spectroscopy (BIS). Changes in body weight were also accounted for before each testing session. Each method (Table 7) determined that all participants were in a euhydrated state before each testing session. Therefore, this eliminates the possibility of dehydration as a

probable cause for the non-significant difference in serum testosterone levels in response to the LV and HV resistance exercise sessions.

Skeletal Muscle Testosterone & DHT

No significant changes in either muscle testosterone or DHT were observed. This is in contrast with previous studies which have shown treadmill running and swimming can result in an increase in muscle testosterone content after an acute exercise bout (Aizawa et al., 2007; Tchaikovsky, Astratenkova, & Basharina, 1986). However, both of these studies were utilizing rat models to measure skeletal muscle testosterone levels. A study in young resistance-trained women (24 ± 5 year) and men (21 ± 1 year) showed no significant increase in muscle testosterone levels (Vingren et al., 2008). Yet, the researchers did not examine serum testosterone levels. In the present study we showed similar results as Vingren *et al.* with the inclusion of serum testosterone levels. Additionally, muscle DHT levels did not significantly change as a result of the exercise bout. Testosterone is converted into DHT by 5α-reductase; however, skeletal muscle has shown minimal activity of this enzyme compared to other tissue types (Hsiao, et al., 2000; Thigpen, et al., 1993; Zouboulis, et al., 2007). With no significant increase in muscle levels of testosterone and little activity of 5α-reductase within skeletal muscle, the lack of significant alteration in muscle DHT level would be expected.

Muscle mRNA Expression

Myostatin mRNA Expression

Several studies have examined the expression of skeletal muscle myostatin mRNA expression after resistance exercise. These studies looked at various time points

ranging from 3, 6, 24, 48, and 72 hours post-exercise in populations ranging from young (20–35 years) to older (60–75 years) adults as well as both men and women (Dennis, et al., 2008; Drummond, et al., 2009; Hulmi, Kovanen, et al., 2008; Kim, et al., 2005; Louis, et al., 2007). In each of these studies, muscle myostatin levels were shown to significantly decrease as a result of the exercise bout. This is contrary to what this study observed. In the present study, we measured four time points: pre, immediately post, 3 hours, and 24 hours post exercise. No significant alterations were seen in either the LV or HV resistance exercise sessions. Of note, while the data was non-significant, the post-exercise mean values in the LV session were all below baseline levels with little variability. However, the mean values of the HV session at 3 hours and 24 hours post-exercise were elevated above baseline with significant variation in the data (Figure 7).

The myostatin gene has been shown to contain an androgen response element within the promoter region which allows for androgens to regulate myostatin expression (Ma, et al., 2001). Thus, testosterone has shown to have an inhibitory effect on myostatin levels (Mendler, et al., 2007; Singh, et al., 2009). We did not show an elevation in serum or muscle testosterone levels which may be a possible cause for the lack of reduction in myostatin levels seen with previous studies. Yet, a study by Kvorning *et al.* (2007) suppressed testosterone levels through use of GnRH analogue goserelin. Myostatin mRNA expression was still significantly reduced post-exercise (Kvorning et al., 2007). Therefore, our results may indicate another possible mechanism for the non-significant reduction in myostatin mRNA expression as compared to previous studies.

MGF mRNA Expression

Skeletal muscle contains a splice variant of IGF-1 which is known as IGF-1EC or MGF (Barton, 2006; Hameed, et al., 2003; McKay, et al., 2008). The upstream promoter of the IGF-1 gene has two identified androgen response elements indicating testosterone has the ability to up-regulate IGF-1 gene expression (Wu, et al., 2007). The present study showed no significant changes in skeletal muscle MGF mRNA expression. Research by Mauras *et al.* (1998) has shown a decrease in MGF mRNA expression after 10 weeks of testosterone suppression (Mauras et al., 1998). Yet, other research has shown a significant increase in muscle MGF expression after resistance exercise, even with no change in serum testosterone or suppression of testosterone (Bamman, et al., 2001; Kvorning, et al., 2007). MGF is responsive to mechanical stress which can account for the increase in expression even if testosterone levels are blunted (Hameed, et al., 2003). While we did not show significant increases in MGF expression, of note is the mean levels and large variability seen at the 3-hour and 24-hour post-exercise time points for the HV session when compared to the LV session (Figure 8). The results are contrary to the previously referenced studies. Therefore, MGF mRNA expression may have been reduced by another mechanism not examined within this study.

Androgen Receptor

AR mRNA Expression & Protein Content

The AR is known to be responsive (up-regulated) to elevation in testosterone levels both from endogenous and exogenous sources (Carson, et al., 2002; Ferrando, et al., 2002; Kadi, et al., 1999; W. J. Lee, McClung, et al., 2003). The AR has also shown

to be responsive to resistance exercise in a phasic response, with an initial down regulation followed by up-regulation of the receptor (Hulmi, Ahtiainen, et al., 2008; Ratamess, et al., 2005; Spiering, et al., 2009; Vingren, et al., 2009; Vingren, et al., 2010; Willoughby & Taylor, 2004). Contrary to these studies we did not show any significant changes in mRNA expression or protein content for the AR. However, noteworthy is that the HV session at 24 hours post-exercise did have a mean elevation with high variability for mRNA expression (Figure 9). The AR protein content had a mean elevation at 3 hours post-exercise within both resistance exercise sessions; however, with high variability within the data the results were non-significant.

Yet, all studies have not shown a response in the AR to resistance exercise. When testosterone was suppressed with GnRH analogue goserelin no significant changes in AR mRNA expression were seen with resistance exercise (Kvorning, et al., 2007). Our results were similar to Kvorning *et al.* with no significant alteration in AR mRNA expression or protein content. This may be due to the lack of endogenous elevation in serum testosterone as seen with previous studies (Migiano et al., 2010; Nindl, et al., 2001; Simão et al., 2013; Wilkinson, Tarnopolsky, Grant, Correia, & Phillips, 2006).

Conclusion

The purpose of this study was to examine the increase in serum testosterone as a result of an upper-lower body resistance exercise protocol compared to a lower-body only exercise protocol. The study was designed to examine if an endogenous increase in testosterone would affect muscle testosterone, DHT, and the AR. The secondary purpose was to examine any interaction with myostatin and MGF. Unexpectedly, we did not see a rise in serum testosterone levels even though the protocol was based upon previous

established guidelines. Therefore, we conclude that higher-intensity resistance exercise of the upper-body performed immediately prior to lower-body resistance exercise does not result in a significant elevation in blood testosterone in resistance-trained participants. We also conclude that this lack of increase in endogenous testosterone has no impact of muscle testosterone, DHT, and the AR protein, which subsequently are all unable to effect the transactivation of the AR, myostatin and MGF.

There are a number of possibilities for the incongruence between the results presented herein and those discussed from the literature. One obvious possibility is that the sample size was small. Another possibility lies within the resistance training status of our population. Our population was extensively resistance-trained compared to other studies which utilized either untrained or minimally-trained individuals. However, a study by Kraemer *et al.* (1992) in elite junior weightlifters showed subjects with > 2 years of training experience had a significantly greater exercise-induced increase in serum testosterone response when compared to untrained subjects (W. Kraemer et al., 1992). Tremblay *et al.* (2004) also showed resistance-trained subjects had a significantly higher total and free testosterone response one hour post-acute resistance exercise when compared to either sedentary or aerobically-trained individuals (Tremblay, Copeland, & Van Helder, 2004). Cadore *et al.* (2008) showed strength-trained individuals with > 3 years of training had a significantly higher free testosterone response, but not total testosterone, after an acute resistance exercise bout when compared to untrained men (Cadore, et al., 2008). These data suggest that the resistance training status may actually increase endogenous testosterone response to resistance exercise (Cadore & Kruel). However, other studies have shown no significant difference between strength-trained or

untrained individuals. The present study utilized individuals whom were extensively trained which had the potential to result in a higher total or free testosterone response compared to a non-resistance trained population. Yet, we still showed no significant increase in serum testosterone levels.

Acute hormonal responses with different types of fatiguing, heavy-resistance exercise with multiple versus single sets have shown the higher total work load to result in a greater endocrine response (Gotshalk et al., 1997; Hakkinen & Pakarinen, 1993). The increased hormonal response does not only take into account testosterone, but other endocrine hormones that may have an inhibitory effect on testosterone levels. Nindl *et al.* (2001) showed a blunted testosterone response after a single bout of heavy resistance exercise in young men who were categorized as above average in both aerobic and anaerobic fitness. The exercise bout consisted of 50 total sets consisting of squats, bench press, leg press, and latissimus dorsi pull downs. However, the researcher also showed cortisol to be significantly elevated for up to two hours post exercise (Nindl, et al., 2001). It has been shown that high pharmacological levels of cortisol will have a significant inhibition on circulating serum testosterone concentrations (Bambino & Hsueh, 1981; Cumming, Quigley, & Yen, 1983). A negative relationship has been observed between circulating cortisol and testosterone levels post-resistance exercise (Brownlee, et al., 2005). Thus, the higher the circulating cortisol levels the lower the total testosterone response. Glucocorticoids (cortisol) are also known to inhibit the expression of skeletal muscle IGF-1 and promote the expression of myostatin (Rennie, Wackerhage, Spangenburg, & Booth, 2004). The present study utilized high total work during the exercise bout in the upper-lower body protocol. Furthermore, “forced” repetitions were

employed to complete the targeted number of repetition for each set when the subjects failed to do so. All subjects failed during each of the upper body exercises (bench press, seated rows, shoulder press). Each subject failed before the goal of 10 repetitions in either the third or fourth set. Kraemer *et al.* (1999) demonstrated no significant increase in cortisol after a single bout of heavy resistance exercise in trained power lifters (W. J. Kraemer, Fleck, et al., 1999). During the current study the lower-body only group performed only leg extensions which most likely did not elicit a cortisol response. Yet, the same group performing the upper-lower body program may have experienced a significant cortisol response due to the strenuous nature of the exercise protocol which may have accounted for non-significant findings with post exercise serum testosterone levels. While measuring cortisol levels was beyond the scope of this study, heart rate measurements did show a significant increase immediately post exercise and for up to three hours after the exercise protocol both within and between groups (Figure 2). Additional research is needed to examine systemic and local hormonal and molecular responses when high total work is utilized with "forced" repetitions in an extensively-trained population.

APPENDICES

APPENDIX A

BAYLOR UNIVERSITY

Department of Health, Human Performance, & Recreation

Informed Consent Form

Title of Investigation: Effects of Endogenous Elevations in Testosterone on Resistance Exercise-Induced Skeletal Muscle Androgen Receptor –Mediated Signaling and DNA Binding in Men

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Sponsors: Exercise and Biochemical Nutrition Lab (Baylor University)

Rationale:

Resistance exercise stimulates skeletal muscle growth which results from protein accumulation within muscle fibers, thus promoting muscle fiber growth. There are a number of physiological mechanisms known to contribute to this process such as intrinsic factors within skeletal muscle, immune/inflammatory, and endocrine (hormonal). Of the endocrine factors, a number of studies have shown the ability of resistance exercise to increase the endogenous level of testosterone. Testosterone is an androgenic hormone with robust growth-promoting capabilities in skeletal muscle due to its ability to increase muscle protein synthesis. Being a fat-soluble hormone, testosterone will bind with its androgen receptor within skeletal muscle. Upon binding, this hormone-receptor complex migrates into the nucleus where it binds to a specific sequence within various muscle-

specific genes, thereby enhancing gene expression. Resistance exercise affects androgen signaling via several mechanisms. First, resistance exercise elevates endogenous testosterone concentrations. Elevations in testosterone due to resistance exercise are known to increase muscle strength following long-term training. Second, muscle contraction increases muscle androgen receptor content due to enhancing androgen receptor gene expression. Third, evidence indicates that elevations in endogenous testosterone following resistance exercise could feasibly increase muscle androgen receptor content independently of the influence of muscle contraction. This process of androgen signaling represents an important target for research aimed at promoting muscle growth and/or counteracting the muscle atrophy characteristic of aging, inactivity/immobilization, and various diseases. Therefore, the purpose of this study is to determine the influence of elevated endogenous circulating testosterone levels on skeletal muscle testosterone, androgen receptor protein, and myofibrillar protein concentration, as well as the mRNA expression of the androgen receptor.

Description of the Study:

I will be one of 10 apparently healthy, non-resistance-trained males between the ages 18 to 30 who will participate in this study. I understand that I will be required to visit the laboratory five times during the course of the study in the following manner: visit 1 = entry/familiarization session, visit 2 = testing/resistance exercise session 1, visit 3 = 24 hour follow-up for session 1, visit 4 = testing/resistance exercise session 2, visit 5 = 24 hour follow-up for session 2. Relative to the 2 testing sessions, I will perform a resistance exercise session involving the knee extension exercise on two occasions separated by three weeks. One session will constitute the control session and be preceded by rest and the other will constitute the experimental session and preceded by a bout of high-volume, moderate-intensity upper-body resistance exercise using short rest periods. At each of the 5 visits, I will have my heart rate and blood pressure assessed. At visits 2 – 5, I will also have blood and muscle samples obtained.

During an initial familiarization session (visit 1), I will be informed of the requirements of the study and sign an informed consent statement in compliance with the Human Subjects Guidelines of Baylor University and the American College of Sports Medicine. A trained individual will examine me to determine if I am qualified to participate in this study. If I am cleared to participate in the study, I will be familiarized to the testing procedures and will then undergo assessments for body composition and muscle strength. This session will take approximately 60 minutes to complete. Once I complete the familiarization session, I will be scheduled for the first resistance exercise session (visit 2).

During the familiarization session, I understand that I will have my maximum muscle strength determined on the bench press, seated row, overhead shoulder press and knee extension exercises. I will warm up by completing 5 to 10 repetitions with a very light weight and then complete 3 to 5 repetitions with a heavier weight. The weight will then be increased conservatively, and I will attempt to lift the weight for one repetition. If the

lift is successful, I will rest for 2 minutes before attempting the next weight increment. This procedure will be continued until I fail to complete the lift. My maximum strength will be recorded as the maximum weight I am able to lift for one repetition. In addition, I will have my body composition (body fat and muscle mass) determined using dual-energy x-ray absorptiometry (DEXA). The DEXA body composition test will involve me lying down on my back in a comfortable position in a pair of shorts/t-shirt. A low dose of radiation will then scan my entire body for approximately 6 minutes. Radiation exposure from the DEXA is approximately 1.5 mR per scan. This is similar to the amount of natural background radiation I would receive in one month while living in Waco, TX. The maximal permissible x-ray dose for non-occupational exposure is 500 mR per year. Total radiation dose will be less than 5 mR for the entire study.

Following the familiarization session, I will be instructed to refrain from exercise for 48 hours and fast for 8 hours prior to each resistance exercise session. I will be provided with a dietary analysis form that I am to complete for 4 days prior to each resistance exercise testing session. Once I report to the lab for each testing session, I will turn in my dietary analysis form.

I understand that I will be required to participate in a 2 separate testing sessions (visits 2 & 4) consisting of the knee extension exercise where I will perform 5 sets of 5 repetitions at 90% - 95% of my maximum strength. However, for one session I will perform 4 sets of 10 repetitions using 80% of my maximum strength on the bench press, seated row, and overhead shoulder press exercises immediately before the knee extension exercise.

During the study, I understand that I will donate about 20 milliliters (4 teaspoons) of venous blood from a vein in my arm by way of an intravenous catheter inserted into my arm using sterile techniques by an experienced phlebotomist using standard procedures. This procedure may cause a small amount of pain when the needle is inserted into the vein as well as some bleeding and bruising, and will remain in place until the end of the testing process. However, proper pressure will be applied upon removal to reduce bruising. I understand that I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn. I understand that personnel who will be inserting the intravenous catheter and taking my blood are experienced in phlebotomy (procedures to take blood samples) and are qualified to do so under guidelines established by the Texas Department of Health and Human Services. The process of inserting the catheter and blood draws at each sampling point will take about 5 minutes and I will be required to leave the catheter in place for the duration of the testing session. Throughout the testing, the catheter will be securely placed in my arm; however, during the 3 hours following resistance exercise I may read or participate in other sedentary activities. At visits 2 and 4 (resistance exercise/testing sessions 1 and 2) I will have blood obtained through the catheter at 8 different time points. However, 24 hours after each session (visits 3 and 5) I will have blood sampled through a standard venipuncture.

In addition to the blood draws, I will undergo the muscle biopsy. I understand that I will have the biopsy location identified on the thigh (opposite thigh for the second exercise session). The biopsy area will be shaved clean of leg hair, washed with antiseptic soap and cleaned with rubbing alcohol. In addition, the biopsy site will be further cleansed by swabbing the area with Betadine (fluid antiseptic). I understand that a small area of the cleaned skin approximately 2 cm in diameter will be anesthetized with a 1.0 mL subcutaneous injection of the topical anesthetic Lidocaine. Once the local anesthesia has taken effect (approximately 2-3 minutes) the biopsy procedure will only take 15-20 seconds. Once anesthetized, I understand that a scalpel point will be used to make an incision approximately 1 cm in length through the skin. Due to the localized effects of the anesthetic, however, I should feel no pain during this process. At this point, I understand that the biopsy needle will be advanced into the incision approximately 1 cm and during this part of the procedure I may feel pressure in my thigh area. Once the muscle sample has been obtained, pressure will be immediately applied to the incision. With the incision being so small bleeding is slight; therefore, only a butterfly bandage is needed to close the incision, which will then be covered with a pressure bandage. I understand that I will be provided verbal and written instructions for post-biopsy care. I understand that I will be instructed to leave the butterfly bandage in place for 72 hours. However, I understand that I will be asked to report back to the lab 24 hours after the biopsy (unless unexpected bleeding or pain occurs) to have the old bandage removed, the incision inspected and new bandages applied, and that I will also report back to the lab for the same reason at 48 hours after the biopsy. I will be further advised to refrain from vigorous physical activity with my leg during the first 24 hours post-biopsy. I understand that if I feel it necessary I may take a non-prescription analgesic medication such as acetominophen to relieve pain if needed and that some soreness of the area may occur for about 24 hours after the biopsy. I will also be advised to avoid such medications such as aspirin, Advil, Bufferin, Nuprin, or Ibuprofen as they may lead to bruising at the biopsy site. I understand that I will be asked to undergo the muscle biopsy procedure on 8 separate occasions throughout the study.

I understand that when I report to the laboratory for the two testing/resistance exercise sessions on visits 2 and 4, I will turn in my 4-day dietary records. In addition, I will have my heart rate and blood pressure determined, and will also provide blood and muscle samples. I understand that if clinically significant side effects are reported from my participation in the study, I will be referred to discuss the problem with Darryn Willoughby, Ph.D. Upon his discretion, I may be referred to discuss the matter with my primary care physician to determine whether any medical treatment is needed and/or whether I can continue in the study. I understand that if I fail to report my progress and health status to the research assistant I may be removed from the study.

I agree to do my best to: 1) follow the instructions outline by the investigators; 2) show up to all scheduled testing times; and 3) put forth my best effort as instructed. I agree not to take any other nutritional supplements or performance enhancing aids during this study (i.e. vitamins/minerals, creatine, HMB, androstenedione, DHEA, etc). In addition, I agree not to take any non-medically prescribed medications and to report any medication that is prescribed for me to take during this study. I understand that if I take any nutritional supplements or medications during the course of the study that I will be removed from the study.

Exclusionary Criteria

I understand that in order to participate in the study, a trained individual will examine me to determine whether I qualify to participate. I understand that I will not be allowed to participate in this study if: 1.) I have any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism; 2.) I have a history of pulmonary disease, hypertension, liver or kidney disease, musculoskeletal disorders, neuromuscular or neurological diseases, autoimmune disease, cancer, peptic ulcers, or anemia; 3.) I am taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (ie, thyroid, insulin, etc), psychotropic, neuromuscular/neurological, or androgenic medications; 4.) I have any bleeding disorders; 5.) I have any chronic infections (e.g., HIV); 6) I have a known allergic reaction to topical anesthetics.

I have reported all nutritional supplements, medically prescribed drugs, and non-medically prescribed drugs that I am presently taking. I have reported whether I have had any prior allergic reactions to topical anesthetics. I have completed medical history questionnaires and am not aware of any additional medical problems that would prevent me from participating in this study. I agree to report all changes in medical status, nutritional and/or pharmacological agents (drugs) that I take during the course of the investigation to Darryn Willoughby, Ph.D. (254-710-3504). I understand that if I experience any unexpected problems or adverse events from participating in this study I may be referred to discuss the problem with my primary care physician to determine whether any medical treatment is needed and/or whether I can continue in the study.

Risks and Benefits

I understand that there are minor risks of muscular pain and soreness associated with the resistance training protocol required in this study which are not uncommon to any exercise program especially for individuals who do not resistance train on a regular basis. On 16 separate occasions during this study, I understand that I will have approximately 4 teaspoons (20 milliliters) of blood drawn from a vein in my forearm. On 14 of these occasions, blood will be obtained using an in-dwelling venous catheter, whereas on 2 other occasions it will involve a standard blood draw using a sterile needle. All blood sampling will be performed by an experienced phlebotomist following an 8-hour fast. This procedure may cause a small amount of pain when the needle is inserted into my

vein as well as some bleeding and bruising. I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn.

On 8 separate occasions during this study (4 at each testing/exercise session), I understand that I will undergo a muscle biopsy in which a small sample of muscle will be obtained from the thigh of my exercised leg. I understand that Darryn Willoughby, Ph.D. or Mike Spillane, M.S.Ed. will perform all of the biopsies and that a local anesthetic (Lidocaine) will be injected into the skin of my thigh prior to the biopsy, which will help prevent any pain and discomfort during the procedure. I understand that I will have a small incision made in my skin and a biopsy needle introduced 1 cm into the incision. I also understand that the incision is so small that it will not require any stitches and will be simply closed with a butterfly bandage and then covered with an adhesive bandage (band-aid). After the anesthetic wears off within 3-4 hours, I understand that the sensation at the biopsy site is comparable to that of a bruise and may persist for 24-36 hours after the procedure. I understand that I am required to inform the study investigators if I have had any prior allergic reactions to anesthesia (e.g. while in the hospital or during a dental visit).

Alternative Treatments

This is not a medical treatment. Therefore, if medical treatment is needed, I must obtain treatment for any medical problem I might have from my personal physician.

Costs and Payments

If I am a Baylor University student, I will not receive any academic credit for participating in this study. I understand that if I am an intercollegiate scholarship athlete I may not be eligible to receive payment to participate in this study. Eligible participants will be paid $100 for completing the familiarization and experimental testing sessions. I also understand that I will be given free blood assessments during the course of the study as described above and may receive information regarding results of these tests if I desire.

New Information

Any new information obtained during the course of this research that may affect my willingness to continue participation in this study will be provided to me. In addition, I will be informed of any unusual/abnormal clinical findings in which medical referral to my personal physician may be warranted. If I desire, I may request that this information be provided to my physician.

Confidentiality

I understand that any information obtained about me in this research, including medical history, laboratory findings, or physical examination will be kept confidential to the extent permitted by law. However, I understand in order to ensure that FDA regulations are being followed, it may be necessary for a representative of the FDA to review my

records from this study which may include medical history, laboratory findings/reports, statistical data, and/or notes taken about my participation in this study. In addition, I understand that my records of this research may be subpoenaed by court order or may be inspected by federal regulatory authorities. I understand that data derived may be used in reports, presentations, and publications. However, I will not be individually identified unless my consent is granted in writing. Additionally, that confidentiality will be maintained by assigning code numbers to my files, limiting access to data to research assistants, locking cabinets that store data, and providing passwords to limit access to computer files to authorized personnel only. I understand that once blood and muscle samples are analyzed that they will be discarded.

Right to Withdrawal

I understand that I am not required to participate in this study and I am free to refuse to participate or to withdraw from the study at any time. Further, that my decision to withdraw from the study will not affect my care at this institution or cause a loss of benefits to which I might be otherwise entitled. If there is concern about my medical safety, I may be referred to seek medical attention.

Compensation for Illness or Injury

I understand that if I am injured as a direct result of taking part in this study, I should consult my personal physician to obtain treatment. I understand that the cost associated with the care and treatment of such injury will be the responsibility of me or my insurance carrier. In some cases, insurers may not reimburse claims submitted for a research-related injury resulting from medical procedures or treatments performed as part of a research study. I understand that Baylor University, the investigator's institutions, and the grant sponsor have not budgeted funds to compensate me for injury or illness that may result from my participation in this study and thus will not be accountable for illness or injury acquired during the course of this study. However, I may be referred to my personal physician if any clinically significant medical/psychological findings are observed during the course of this study.

I agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss I suffer as a result of my participation in this study regardless of the cause of my injury, damage or loss.

Statement on Conflict of Interest

I understand that this study is funded by the Exercise and Biochemical Nutrition Laboratory at Baylor University, and that the researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Voluntary Consent

I certify that I have read this consent form or it has been read to me and that I understand the contents and that any questions that I have pertaining to the research have been, or will be answered by Darryn Willoughby, Ph.D. (principal investigator, Department of Health, Human Performance & Recreation, 120 Marrs McLean Gymnasium, Baylor University, phone: 254-710-3504) or one of the research associates. My signature below means that I am at least 18 years of age and that I freely agree to participate in this investigation. I understand that I will be given a copy of this consent form for my records. If I have any questions regarding my rights as a research subject in this study, I may contact Baylor's University Committee for Protection of Human Subjects in Research. The Chairman is Dr. Michael Sherr, School of Social Work, P.O. Box 97320, Waco, Texas 76798, phone number (254) 710-4483.

Date _____ Subject's Signature _____

I certify that I have explained to the above individual the nature and purpose of the potential benefits and possible risks associated with participation in this study. I have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

Date _____ Investigator's Signature _____

APPENDIX B

Application to the Baylor IRB

For Review of Research/Activity Proposal
Part 1: Signature Page

1. Name Darryn S. Willoughby, Ph.D., FACSM, FISSN

2. Email Address (optional) Darryn_Willoughby@baylor.edu

3. Complete Mailing Address P.O. Box 97313

4. Position Associate Professor

5. Faculty Advisor (if researcher is Graduate Student) _____

6. Department/School HHPR/SOE & Biomedical Science Institute

7. Telephone # x3504 FAX # x3527

8. Are you using participants in research (***Y*** or N) or in teaching exercises (Y or ***N***)?

9. Title of the research project/teaching exercise: **Effects of Endogenous Elevations in Testosterone on Resistance Exercise-Induced Skeletal Muscle Androgen Receptor - Mediated Signaling and DNA Binding in Men.**

10. Please return this signed form along with all the other parts of the application and other documentation to the University Committee for Protection of Human Subjects in Research; Dr. Michael Sherr, Chairman, School of Social Work, P.O. Box 97320, Waco, Texas 76798, phone number (254) 710-4483. If you have questions, or if you would like to see a copy of the OHRP Report on protection of human subjects in research, contact Dr. Sherr at extension 4483.

02/02/11

Darryn S. Willoughby _____ _____

Signature of Principal Investigator Date

_____

Signature of Faculty Advisor (required if researcher is a Graduate Student)

Departmental Review: _____

Department Chair or the Chair's Designate

Part 2: Introduction & Rationale

Resistance exercise stimulates skeletal muscle growth which results from protein accretion within existing muscle fibers, thus promoting muscle fiber hypertrophy (1). There are a number of physiological mechanisms known to contribute to this process such as intrinsic factors within skeletal muscle, immune/inflammatory, and endocrine. Of the endocrine factors, a number of studies have shown the ability of resistance exercise to increase the endogenous level of testosterone (1, 2, 3, 4). Testosterone is an androgenic hormone with robust anabolic capabilities in skeletal muscle due to its ability to increase muscle protein synthesis. Being a lipophilic hormone, testosterone will bind with its androgen receptor within skeletal muscle. Upon binding, this hormone-receptor complex migrates into the nucleus where it binds to a specific sequence within various muscle-specific genes, thereby up-regulating gene expression. Resistance exercise affects androgen signaling via several mechanisms. First, resistance exercise transiently elevates endogenous testosterone concentrations (2). Transient elevations in testosterone due to resistance exercise are known to increase muscle strength following long-term training (5). Second, muscle contraction up-regulates muscle androgen receptor content due to up-regulation in androgen receptor gene expression (6, 7, 8). Third, evidence indicates that transient elevations in endogenous testosterone following resistance exercise could feasibly increase muscle androgen receptor content independently of the influence of muscle contraction (4). This process of androgen signaling represents an important target for research aimed at promoting muscle growth and/or attenuating the muscle atrophy characteristic of aging, inactivity/immobilization, and various diseases. Therefore, the purpose of this study is to determine the influence of transiently elevated endogenous circulating testosterone levels on skeletal muscle testosterone, androgen receptor protein, and myofibrillar protein concentration, as well as the mRNA expression of the androgen receptor.

Part 3: Methodology

Methods

Experimental Approach

In a randomized, cross-over design, participants will visit the laboratory on 5 separate occasions in the following manner: visit 1 = entry/familiarization session, visit 2 = testing/resistance exercise session 1, visit 3 = 24 hour follow-up for session 1, visit 4 = testing/resistance exercise session 2, visit 5 = 24 hour follow-up for session 2. Relative to the testing sessions (visits 2 & 4), participants will perform a resistance exercise session involving the knee extension exercise on two occasions separated by three weeks. One session will constitute the control session and be preceded by rest and the other will

be preceded by the experimental session and preceded by a bout of high-volume, moderate-intensity upper-body resistance exercise using short rest periods (Figure 1). This approach is based on the premise that the resistance exercise session immediately preceded by upper-body resistance exercise will elevate endogenous testosterone compared to the resistance exercise session preceded by rest (4). This will help to determine if endogenous elevations in testosterone will be involved in regulating androgen receptor gene and protein expression due to facilitating androgen-receptor mediating up-regulations in DNA binding capacity.

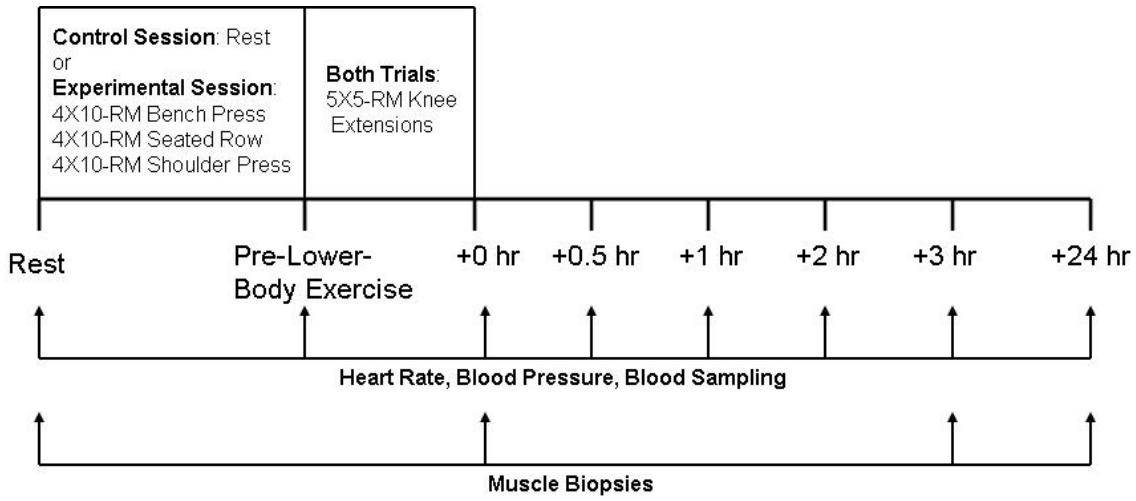

Figure 1. An illustration of the experimental protocol to be used in the study. At each of the 8 time points, heart rate and blood pressure will be assessed and blood samples will be obtained. Muscle biopsies, however, will be obtained at rest, +0 hr, +3 hr, and +24 hr.

Participants

Ten apparently healthy, recreationally active, but non-resistance trained [no regular, consistent resistance training (i.e. thrice weekly) for at least 1 year prior to the onset of the study], men between the ages of 18-30 will volunteer to serve as participants in this study. Enrollment will be open to men of all ethnicities. Only participants considered as low risk for cardiovascular disease and with no contraindications to exercise as outlined by the American College of Sports Medicine (ACSM), and who have not consumed any nutritional supplements (excluding multi-vitamins) 3 months prior to the study will be allowed to participate. All eligible subjects will sign university-approved informed consent documents and approval will be granted by the Institutional Review Board for Human Subjects. Additionally, all experimental procedures involved in the study will conform to the ethical consideration of the Helsinki Code.

Study Site

All supervised testing and supplement assignment will be conducted in the Exercise & Sport Nutrition Laboratory (ESNL) at Baylor University. All sample analyses will be completed in the Exercise and Biochemical Nutrition Laboratory (EBNL) at Baylor University.

Independent and Dependent Variables

The independent variable will be the resistance exercise protocol (control vs. experimental). Dependent variables in serum will include: free and total testosterone. In skeletal muscle, the variables will include: testosterone, dihydrotestosterone, androgen receptor mRNA expression, androgen receptor protein expression, androgen receptor/DNA binding capacity, and myofibrillar protein content.

Entry and Familiarization Session (Visit 1)

Participants expressing interest in participating in this study will be interviewed on the phone to determine whether they appear to qualify to participate in this study. Participants believed to meet eligibility criteria will then be invited to attend an entry/familiarization session. Once reporting to the lab, participants will complete a medical history questionnaire and undergo a general physical examination to determine whether they meet eligibility criteria. Participants meeting entry criteria will be familiarized to the study protocol via a verbal and written explanation outlining the study design and will undergo assessments for body composition and muscle strength assessments. At the conclusion of the familiarization session, participants will be given an appointment in which to attend their first testing session. In addition, each participant will be instructed to refrain from exercise for 48 hours, fast for 8-hours, and record their dietary intake for 4 days prior to each of the two testing sessions involved in the study.

Muscle Biopsies (Exercise Session 1/Visit 2 & Exercise Session 2/Visit 4, Visits 3 & 5)

Percutaneous muscle biopsies (50-70 mg) will be obtained from the middle portion of the vastus lateralis muscle of the dominant leg at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm. After the initial biopsy, for the remaining biopsies attempts will be made to extract tissue from approximately the same location as the initial biopsy by using the pre-biopsy scar, depth markings on the needle, and a successive incision that will be made approximately 0.5 cm to the former from medial to lateral. After removal, adipose tissue will be trimmed from the muscle specimens and will be immediately frozen in liquid nitrogen and then stored at -80°C for later analysis. Four muscle samples will be obtained at each of the two resistance exercise sessions, with a total of eight muscle samples being obtained during the course of the study. At each testing session, muscle samples will be obtained: immediately prior to commencing the testing session, immediately after lower-body exercise, 3 hours after exercise, and 24 hours after exercise.

Blood Sampling (Exercise Session 1/Visit 2 & Exercise Session 2/Visit 4, Visits 3 & 5)

Venous blood samples will be obtained into 10 ml vacutainer tubes from a 20 gauge intravenous catheter inserted into the antecubital vein. Blood samples will be allowed to

stand at room temperature for 10 min and then centrifuged. The serum will be removed and frozen at -80°C for later analysis. Eight blood samples will be obtained at each of the two resistance exercise sessions, with a total of 16 blood samples being obtained during the course of the study. At each testing session, blood samples will be obtained: immediately prior to the commencing the testing session, immediately prior to lower-body exercise, immediately after lower-body exercise, 0.5 hour after exercise, 1 hour after exercise, 2 hours after exercise, and 3 hours after exercise. However, 24 hours after the exercise session blood venous blood samples will be obtained from the antecubital vein into a 10 ml collection tube using a standard Vacutainer apparatus.

Body Composition Testing (Entry and Familiarization Session/Visit 1)

At the entry and familiarization session, total body mass (kg) will be determined on a standard dual beam balance scale (Detecto). Total body water (total, intracellular, and extracellular) will be determined with bioelectrical impedance [(BIA) Xitron 4200, San Diego, CA]. Percent body fat, fat mass, and fat-free mass, will be determined using using a calibrated Hologic 4500W dual-energy x-ray absorptiometry (DEXA). The DEXA will segment regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments.

Dietary Analysis (Exercise Session 1/Visit 2 & Exercise Session 2/Visit 4)

Participants will be required to record their dietary intake for 4 days prior to each of the two resistance exercise sessions. The participants' diets will not be standardized and participants will be asked not to change their dietary habits during the course of the study. The dietary recalls will be evaluated with the Food Processor dietary assessment software program to determine the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet for the duration of the study.

Muscle Strength Assessments (Entry and Familiarization Session/Visit 1)

In order to determine muscular strength, participants will perform one-repetition maximum (1-RM) tests on the bench press, seated row, overhead shoulder press and knee extension exercises while attending the familiarization session. Participants will warm up by completing 5 to 10 repetitions at approximately 50% of the estimated 1-RM. The participant will rest for 1 minute, and then complete 3 to 5 repetitions at approximately 70% of the estimated 1-RM. The weight will then be increased conservatively, and the participant will attempt to lift the weight for one repetition. If the lift is successful, the participant will rest for 2 minutes before attempting the next weight increment. This procedure will be continued until the participant fails to complete the lift. The 1-RM will be recorded as the maximum weight that the participant is able to lift for one repetition.

Resistance Exercise Protocol (Exercise Session 1/Visit 2 & Exercise Session 2/Visit 4)

During the control session, participants will perform 5 sets of 5-RM (90%-95% 1-RM) of the bilateral knee extension exercise with 3 minutes of rest between sets. This heavy-load knee extension protocol will involve low total work and long rest periods which has been previously shown to induce minimal increases in testosterone response (2, 4). However, during the experimental session, participants will perform in the following order, an upper-body resistance exercise protocol of 4 sets of 10-RM each of the bench press, seated row, and overhead shoulder press exercises immediately prior to the knee extension protocol (3, 4). For the upper-body protocol, the initial load will be set at 80% 1-RM for each participant. If muscle fatigue/failure occurs during a set, a spotter will provided assistance until the participant completes the remaining repetitions and resistance will be reduced for subsequent sets. In all cases, 2 minutes of rest will separate sets and exercises. Thigh high-volume, short rest period, upper-body protocol has been shown to maximize endogenous testosterone levels in response to resistance exercise (2, 4). Within 2 minutes, participants will begin the knee extension exercise protocol identically as performed during the control trial. All training sessions will be conducted in the ESNL at Baylor University and supervised by study personnel.

Assessment of Heart Rate & Blood Pressure (Visits 1, 2, 3, 4, & 5)

At visits 1-5, heart rate and blood pressure will be assessed. At the entry and familiarization session, these variables will be obtained as part of the health history assessment. At visits 2 and 4, heart rate and blood pressure will be obtained at each of the 7 time point where blood samples are obtained. Heart rate and blood pressure will also be obtained at visits 3 and 5. Heart rate will be determined by palpation of the radial artery using standard procedures. Blood pressure will be assessed in the supine position after resting for 5-min using a mercurial sphygmomanometer using standard procedures.

Serum Free and Total Testosterone Assessments

From the 16 total blood samples obtained from the 2 resistance exercise sessions, total and free testosterone levels will be determined using commercially available enzyme-linked immunoabsorbent assays (ELISA) kits (Diagnostic Systems Laboratories, Webster, TX) with a microplate reader (Wallac Victor 1420, Perkin Elmer, Boston MA). All samples will be run in duplicate and the assays will be performed at 450 nm wavelength, each against a known standard curve. Data analysis will be performed using MicroWin microplate data-reduction software (Mikrotek Laborsysteme, Germany).

Skeletal Muscle Testosterone and DHT Assessments

From the 8 muscle samples obtained at the 2 resistance exercise sessions, free testosterone and DHT levels will be determined using commercially available enzyme-linked immunoabsorbent assays (ELISA) kits (Diagnostic Systems Laboratories,

Webster, TX) with a microplate reader (Wallac Victor 1420, Perkin Elmer, Boston MA). All samples will be run in duplicate and the assays will be performed at 450 nm wavlength, each against a known standard curve. Data analysis will be performed using MicroWin microplate data-reduction software (Mikrotek Laborsysteme, Germany).

Assessment of Skeletal Muscle Androgen Receptor mRNA Expression

From the 8 muscle tissue samples obtained at the 2 resistance exercise sessions, the mRNA expression of the androgen receptor gene will be performed using real-time PCR based on our previously established guidelines (8). Oligonucleotide primers will be designed using Primer Express from known human mRNA sequences available online through the NCBI database. The quantity of mRNA will be determined relative to the expression of β-actin, and ΔC_T values will be used to compare gene expression. The specificity of the PCR will be demonstrated with an absolute negative control reaction containing no cDNA template, and single gene products confirmed using DNA melt curve analysis.

Assessment of Skeletal Muscle Androgen Receptor Protein Expression

From the 8 muscle tissue samples obtained at the 2 resistance exercise sessions, activated androgen receptor protein will be determined by a transcription factor ELISA (Active Motif, Carlsbad, CA) which utilizes a consensus sequence of the androgen response element located within the promoter of the androgen receptor gene and a specific polyclonal antibody for the androgen receptor (Santa Cruz Biotech, Santa Cruz, CA) and using ELISA. All samples will be run in duplicate and the assays will be performed at 450 nm wavelength. Data analysis will be performed using MicroWin microplate data-reduction software (Mikrotek Laborsysteme, Germany), and the final concentration expressed relative to muscle wet-weight.

Assessment of Skeletal Muscle Myofibrillar Protein Content

The content of myofibrillar protein from each muscle sample will be determined spectrophometrically at a wavelength of 595 nm using bovine serum albumin as the standard. The final concentration will be expressed relative to muscle wet-weight (8).

Statistical Analyses

Statistical analyses will be performed by utilizing separate 2 x 4 (Session x Test) factorial analyses of variance (ANOVA) with repeated measures. Further analysis of the main effects will be performed by separate one-way ANOVAs. Significant between-group differences will then be determined involving the Tukey's Post Hoc Test. All statistical procedures will performed using SPSS 16.0 software and a probability level of < 0.05 will be adopted throughout.

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Research Team

Darryn S. Willoughby, PhD, FACSM, FISSN, CSCS, CISSN. Dr. Willoughby is an Associate Professor of Exercise and Muscle Physiology and Biochemistry in the Department of Health, Human Performance, & Recreation at Baylor University. He is also an Associate Professor of Baylor's Biomedical Science Institute. Dr. Willoughby is an internationally recognized exercise biochemist and molecular physiologist. He has

conducted a vast amount of research focusing on the biochemical and molecular regulatory mechanisms regarding exercise performance and nutrition. Dr. Willoughby will be the principal supervisor of the project. He will perform blood sampling and muscle biopsies, and oversee all aspects of the study and perform the majority of the biochemical and clinical chemistry assays involved in the project.

Pete Grandjean, PhD, FACSM, CSCS. Dr. Grandjean is an Associate Professor of Exercise Physiology in the Department of Health, Human Performance, & Recreation at Baylor University. Dr. Grandjean will assist in providing oversight in data collection, strength and body composition testing, and performing blood draws and clinical laboratory assessments.

Brian Leutholtz, Ph.D., FACSM. Dr. Leutholtz is a Professor of Exercise Physiology in the Department of Health, Human Performance, & Recreation at Baylor University. Dr. Leutholtz will assist in providing oversight, in data collection, strength and body composition testing, and performing blood draws and clinical laboratory assessments.

Mike Spillane, M.S.Ed. Mr. Spillane is an exercise physiologist pursuing his Ph.D. in Exercise, Nutrition, and Preventative Health and serves as a research assistant in the EBNL. He will perform muscle biopsies and blood draws and assist in all areas involved in the project.

Neil Schwarz, M.S. Mr. Schwarz is an exercise physiologist pursuing his Ph.D. in Exercise, Nutrition, and Preventative Health and serves as a research assistant in the EBNL. He will perform blood draws and assist in all areas involved in the project.

Procedures

Medical Monitoring. Interested participants will be invited to familiarization sessions. During this time, participants will sign consent forms and complete medical history information. Participants will then undergo a general exam to determine whether the subject meets entry criteria to participate in the study. This exam will include evaluating the medical and training history questionnaires and performing a general physical examination according to ACSM exercise testing guidelines. Based on this examination, participants will be assessed for their risk of cardiovascular disease and contraindications to exercise and then a recommendation will be made on whether the participant meets entry criteria and may therefore participate in the study. Trained, non-physician exercise specialists certified in CPR will supervise participants undergoing testing and assessments. A telephone is in the laboratory in case of any emergencies, and there will be no less than two researchers working with each participant during testing sessions. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance. Participants will be informed to report any unexpected problems or adverse events they may encounter during

the course of the study to Darryn S. Willoughby, Ph.D. If clinically significant side effects are reported, the participants will be referred to their physician for medical follow-up. New findings and/or medical referrals of unexpected problems and/or adverse events will be documented, placed in the participants research file, and reported to the Baylor IRB committee.

Screening for Cardiopulmonary Disease Risk and Exercise Contraindications. All participants will have their risk of cardiopulmonary disease and their possible contraindications to exercise assessed by Certified Exercise Physiologists and the laboratory nurse in accordance to standard procedures described by the American College of Sports Medicine (ACSM) (*ACSM's Guidelines for Exercise Testing and Prescription, 8th ed. Williams & Wilkins Publishers, 2010).* Only those participants considered as low risk for cardiovascular disease with no contraindications to exercise will be considered as eligible to participate in the study. These guidelines are outlined and presented below:

ACSM Risk Stratification Criteria for Cardiovascular Disease

Low Risk

Younger individuals (men < 45 years of age; women < 55 years of age) who are asymptomatic for cardiovascular disease and possess no more than one positive cardiovascular disease risk factor.

Moderate Risk

Older individuals and/or those who are asymptomatic for cardiovascular disease and possess no more than two cardiovascular disease risk factors.

High Risk

Individuals who have know cardiovascular, pulmonary, or metabolic disease or one or more signs/symptoms suggestive of such disease.

ACSM Criteria for Signs and Symptoms Suggestive of Cardiovascular Disease

1. Pain, discomfort in the chest, neck, jaw, arms, or other areas that may be due to myocardial ischemia.

2. Shortness of breath at rest or with mild exertion.

3. Dizziness or syncope.

4. Orthopnea or paroxysmal nocturnal dyspnea.

5. Ankle edema.

6. Palpitations or tachycardia.

7. Intermittent claudication.

8. Known heart murmur.

9. Unusual fatigue or shortness of breath with usual activities.

ACSM Absolute and Relative Contraindications to Exercise

Absolute Contraindications

1. Unstable angina.
2. Uncontrolled dysrhythmias.
3. Recent EKG changes and cardiac events.
4. Acute myocarditis or pericarditis.
5. Acute pulmonary embolism or acute myocardial infarction.
6. Severe aortic stenosis.
7. Dissecting aneurysm.
8. Acute infections.

Relative Contraindications

1. Left main coronary stenosis.
2. Severe hypertension (> 200/115).
3. Tachycardia or bradycardia.
4. Uncontrolled metabolic disease.
5. High-degree AV block.
6. Chronic infectious disease.
7. Cardiomyopahty and outflow obstructions.
8. Stenotic valve disease.
9. Ventricular aneurysm.

Assessment of Hemodynamic Safety Markers (Heart Rate & Blood Pressure). Heart rate will be determined by palpation of the radial artery using standard procedures. Blood pressure will be assessed in the supine position after resting for 5-min using a mercurial sphygmomanometer using standard procedures.

Estimated Energy Intake/Dietary Inventories. Participants will record all food and fluid intake for 4 days on dietary record forms for dietary analysis in order to standardize

nutritional intake. Participants will brings these forms with them upon each visit to the laboratory for the two testing sessions. Dietary intake will be assessed using the Food Processor IV Nutrition Software.

Body Composition Assessments. Participants will undergo body composition tests in the ESNL. Prior to each assessment, height will be measured using standard anthropometry and total body weight will be measured using a calibrated electronic scale with a precision of +/- 0.02 kg. Total body water will then be estimated using a Xitron 4200 Bioelectrical Impedance Analyzer (San Diego, CA) which measures bio-resistance of water and body tissues based on a minute low energy, high frequency current (500 micro-amps at a frequency of 50 kHz) transmitted through the body. This analyzer is commercially available and has been used in the health care/fitness industry as a means to assess body composition and body water for over 20 years. The use of this device has been approved by the Food and Drug Administration (FDA) to assess total body water and the current to be used has been deemed safe. This is measured through four electrodes placed on the body: one electrode will be placed on the posterior surface of the right wrist, in between the radial and ulna styloid processes (wrist bones), another electrode will be placed on the posterior surface of the right hand at the distal base of the second metacarpal; the third electrode will be placed on the anterior surface of the right foot at the distal end of the first metatarsal. Participants will lie on a table in the supine position and electrodes will be connected to the analyzer. After the subject is connected, age, gender, weight, height, and activity level are entered into the unit by the technician. After the unit has measured the resistance, which takes approximately 30 seconds, the unit then calculates total body water and body water percent.

Body composition/bone density will then be determined using a calibrated Hologic 4500W dual-energy x-ray absorptiometry (DEXA). The DEXA body composition test will involve having the participant lie down on their back in a standardized position in a pair of shorts/t-shirt. A low dose of radiation will then scan their entire body for approximately six minutes. The DEXA segments regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments for determination of fat, soft tissue (muscle), and bone mass. Radiation exposure from DEXA for the whole body scan is approximately 1.5 mR per scan. This is similar to the amount of natural background radiation a person would receive in one month while living in Waco, TX. The maximal permissible x-ray dose for non-occupational exposure is 500 mR per year. Total radiation dose will be less than 5 mR for the entire study.

Muscle Strength Assessments. Participants will perform one-repetition maximum (1-RM) tests on the bench press, seated row, overhead shoulder press, and knee extension exercises. Participants will warm up by completing 5 to 10 repetitions at approximately 50% of the estimated 1-RM. The subject will rest for 1 minute, and then complete 3 to 5 repetitions at approximately 70% of the estimated 1-RM. The weight will then be

increased conservatively, and the subject will attempt to lift the weight for one repetition. If the lift is successful, the subject will rest for 2 minutes before attempting the next weight increment. This procedure will be continued until the subject fails to complete the lift. The 1-RM will be recorded as the maximum weight that the subject is able to lift for one repetition.

Serum Free and Total Testosterone and Skeletal Muscle Testosterone and Dihydrotestosterone. Serum and muscle samples will be processed and then placed into individual wells of a microtiter plate, previously coated with a primary antibody against each of the respective proteins, for incubation. The plates will then be washed and incubated with a conjugated secondary antibody that binds to the primary antibody. The membrane will be washed again, and then exposed to a chemical substrate that produces color when exposed to the conjugated secondary antibody. Protein concentrations will be determined with a Wallac Victor-1420 micoplate reader (Perkin-Elmer Life Sciences, Boston, MA). The assays will be performed at either 405 or 450 nm wavlength, each against a known standard curve.

Skeletal Muscle Androgen Receptor Expression. Using ELISA, muscle tissue samples will be processed and then placed into individual wells of a microtiter plate, previously coated with a consensus sequence of the androgen response element located within the promoter of the androgen receptor gene and a specific polyclonal antibody for the androgen receptor. All samples will be run in duplicate and the assays will be performed at 450 nm wavelength. Data analysis will be performed using MicroWin microplate data-reduction software (Mikrotek Laborsysteme, Germany), and the final concentration expressed relative to muscle wet-weight.

Myofibrillar Protein Content.

The content of myofibrillar protein from each muscle sample will be determined spectrophometrically at a wavelength of 595 nm using bovine serum albumin as the standard. The final concentration will be expressed relative to muscle wet-weight.

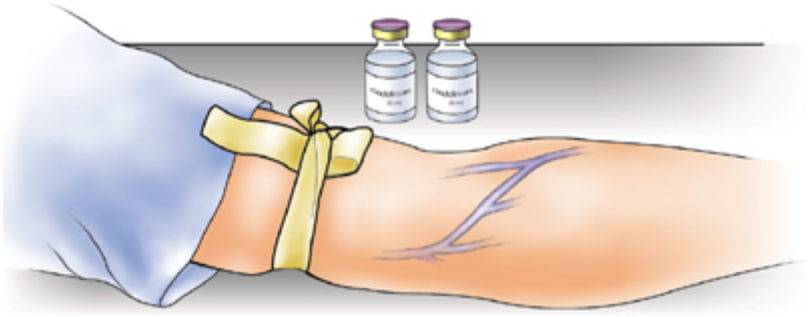
Skeletal Muscle Androgen Receptor mRNA Expression

The mRNA expression of the androgen receptor gene will be performed using real-time PCR. Oligonucleotide primers will be designed using Primer Express from known human mRNA sequences available online through the NCBI database. The quantity of mRNA will be determined relative to the expression of β-actin, and ΔC_T values will be used to compare gene expression. The specificity of the PCR will be demonstrated with an absolute negative control reaction containing no cDNA template, and single gene products confirmed using DNA melt curve analysis.

Blood Samples. Participants will donate approximately 4 teaspoons (20 milliliters) of fasting venous blood during each blood draw. Blood samples will be obtained from an

intravenous catheter placed into the antecubital vein using standard phlebotomy procedures by Darryn Willoughby, Ph.D., Pete Grandjean, Ph.D., Mike Spillane, M.S.Ed., or Neil Schwarz, M.S., who are trained in phlebotomy in compliance with guidelines established by the Texas Department of Health and Human Services. While drawing blood, study personnel will wear personal protective clothing gloves, lab coats, etc.) when handling blood samples. Subjects will be seated in a phlebotomy chair. A tourniquet will be applied high on the brachium (upper arm) and will be tight enough to visibly indent the skin, but not cause the patient discomfort (Figure 2). The entry site will be thoroughly cleaned with an alcohol prep pad and allowed to dry. The entry site will then also be cleaned with betadine swab and allowed to dry (Figure 3). The participant will be instructed to lower their arm and make a fist several times in order to maximize venous engorgement. The appropriate vein will be selected (Figure 4). If a suitable vein is difficult to identify, the pads of the first and second fingers will be used to “slap” the veins gently to help dilate them. Alternately, the arm may be covered with a warm, moist compress to help with peripheral vasodilatation. If after a meticulous search no suitable veins are found, then the tourniquet will be released from above the elbow and placed around the forearm to search in the distal forearm, wrist and hand. If still no suitable veins are found, then the other arm will be checked taking extreme care to stay away from arteries, which are pulsatile. To puncture the vein, the 20 gauge catheter will be held in the dominant hand. With the bevel up, enter the skin at about a 30-degree angle and in the direction of the vein. After entering the skin, the angle of the catheter will be reduced until it is nearly parallel to the skin (Figure 5). If the vein appears to "roll" (move around freely under the skin), the venipuncture will begin by applying counter tension against the skin just below the entry site using the thumb of the non-dominant hand. The skin will be pulled distally toward the wrist in the opposite direction the needle will be advancing, being careful not to press too hard which will compress blood flow in the vein and cause the vein to collapse. The catheter will be advanced into the vein until blood is seen in the "flash chamber" of the catheter. After entering the vein, the plastic catheter (which is over the needle) will be advanced into the vein while leaving the needle stationary (Figure 6). The hub of the catheter will be all the way to the skin puncture site. The tourniquet will be released. Gentle pressure will be applied over the vein just proximal to the entry site to prevent blood flow. The needle will be removed from within the plastic catheter and disposed in an appropriate sharps container (Figure 7). The catheter will be taped in place using the strips of tape and a sterile dressing (Figure 8). Once samples are obtained, the vacutainer holder and needle will be removed. The needle will be discarded as hazardous waste in an appropriately-labeled plastic sharps container. The site of the blood draw will then be cleaned with a sterile alcohol wipe and gauze and a sterile Band-Aid will be placed on the site. The alcohol wipe and gauze then will be discarded in an appropriately-labeled biohazard waste receptacle. The blood collection tubes will be labeled and placed in a test tube rack. Study personnel (all who have received blood

borne pathogen training and will be wearing personal protective clothing) will centrifuge the serum samples, transfer serum into labeled serum storage containers, and store at -80°C for later analysis.



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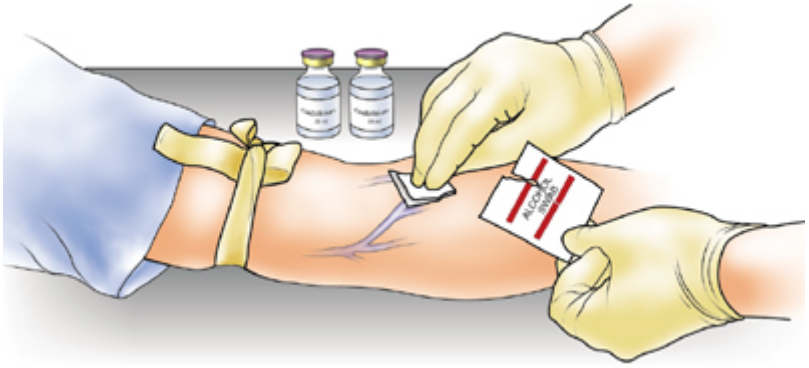

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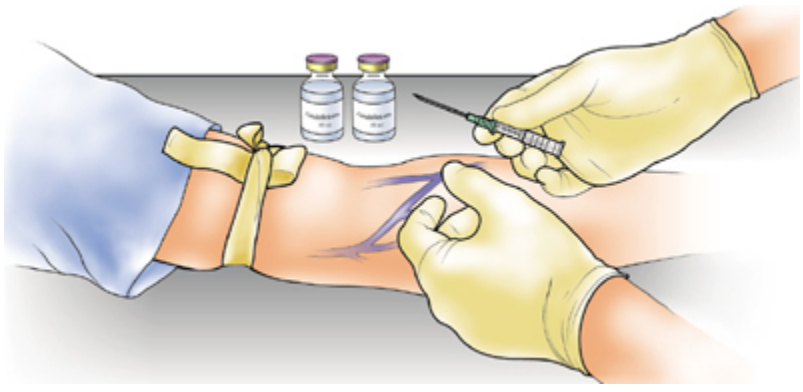

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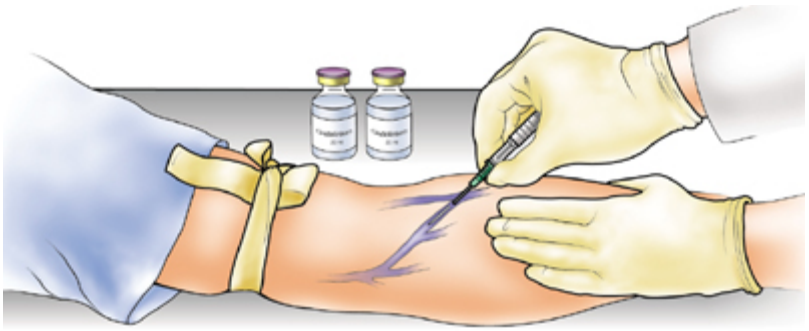

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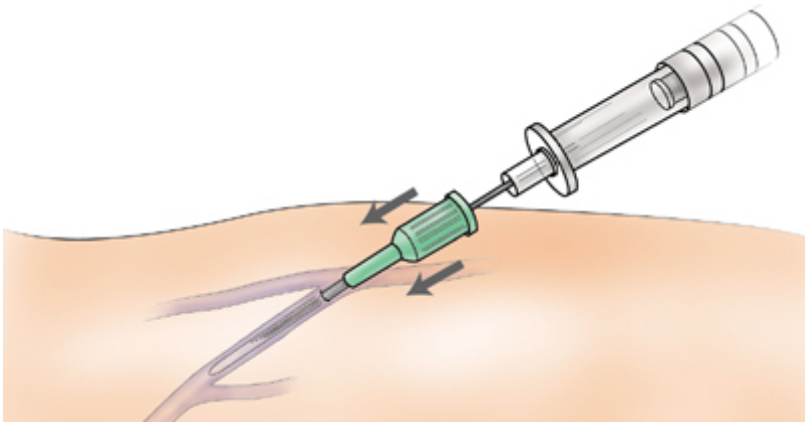

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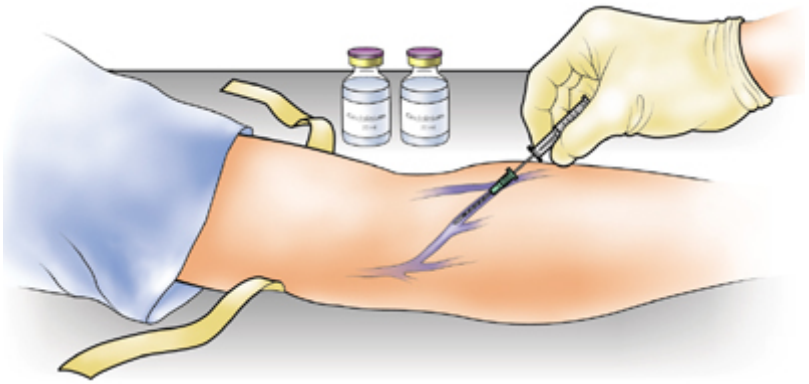

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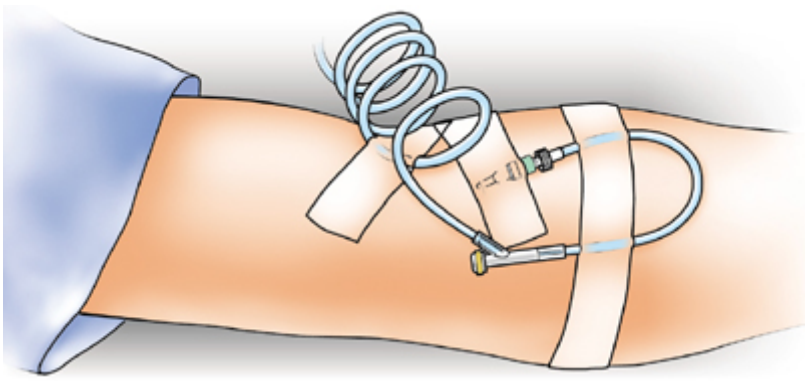
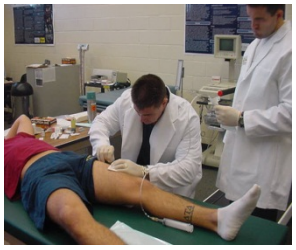


Figure A8.

Muscle Biopsies. Percutaneous muscle biopsies (approximately 50-70 mg) will be obtained from the vastus lateralis of each participant's thigh (Figures 9-12). Samples will be extracted under local anesthesia of 1% Lidocaine from the middle portion of the muscle at the midway between the patella and the greater trochanter of the femur by Darryn Willoughby, Ph.D. or Mike Spillane, M.S.Ed. For each biopsy, muscle tissue will be extracted from the same location by using the previous incision and depth markings on the needle. First, the participant will lie supine or assume a comfortable reclining position on a sterilized table. Once the extraction point is identified, the area will be shaved clean of leg hair, washed with an antiseptic soap and cleaned with rubbing alcohol. In addition, the biopsy site will further be cleansed by swabbing the area with Betadine (fluid antiseptic) and then draped. A small area of the skin approximately 2 cm in diameter will be anesthetized with a 1.0 mL subcutaneous injection of Lidocaine. Once anesthetized, a scalpel point will be used to produce the initial biopsy site by making an incision approximately 1 cm in length through the skin, subcutaneous fat, and fascia. Due to the localized effects of the anesthetic, the participant should feel no pain during this process. The biopsy needle will be advanced into the incision approximately 1 cm and during this part of the procedure the subject may feel pressure to the thigh area. Once the muscle sample has been obtained, pressure will be immediately applied and the wound will immediately be bandaged. With the incision being so small bleeding is slight; therefore, only a butterfly bandage is needed to close the incision, which is then covered with a pressure bandage. The needle and scalpel blade will be discarded as hazardous waste in an appropriately-labeled plastic sharps container. The site of the biopsy will be cleaned with a sterile alcohol wipe and gauze. The alcohol wipe and gauze then will be discarded in an appropriately labeled biohazard waste receptacle. The tissue sample will be stored at -80°C for future analyses. Once the local anesthesia has taken effect (approximately 2-3 minutes) the biopsy procedure will take approximately 15-20 seconds. Written instructions for post-biopsy care will be given to the subjects. The participant will be instructed to leave the bandages on for 24 hours (unless unexpected bleeding or pain

occurs) and asked to report back to the lab within 24 hours to have the old bandages removed, the incision inspected and new bandages applied. The participant will be further advised to refrain from vigorous physical activity during the first 48 hours post-biopsy. These suggestions will minimize pain and possible bleeding of the area. If needed, the subject may take non-prescription analgesic medication such as acetominophen to relieve pain if needed. However, medications such as aspirin, Advil, Nuprin, Bufferin, or Ibuprophen will be discouraged as these medications may lead to ecchymosis at the biopsy site. Soreness of the area may occur for about 24 hours post-biopsy.



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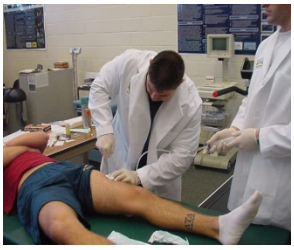

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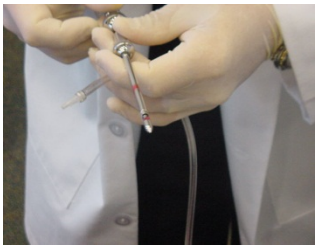


Figure A11.

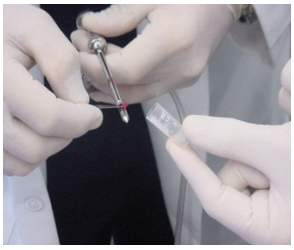


Figure A12.

Equipment

***Digital Scale*.** Total body weight will be determined using a digital scale accurate to ±0.02 kg. The scale is calibrated by placing certified 25-kg weights and balancing the scale. Other than general instructions, special skills are not required to measure body weight.

Mercurial Sphygmomanometer. Blood pressure will be assessed by auscultation of the brachial artery using a mercurial sphygmomanometer using standard clinical procedures.

Bioelectrical Impedance Analyzer (BIA). The Omron HBF-306 Bioelectrical Impedance Analyzer (*Omron Healthcare Inc., Vernon Hills, IL*) which measures bio-resistance and body composition based on a minute low energy, high frequency current transmitted through the body from surface electrodes embedded in the handles of the unit. The analyzer is calibrated internally to a standard electrical current by pressing the calibration key located on the unit. A trained research assistant will perform this procedure.

Dual-Energy X-Ray Absorptiometer (DEXA). Body composition measurements will be determined by qualified personnel (in compliance with State Regulations) using a Hologic Discovery W dual energy x-ray absorptiometer (*Waltman, MA*). This system segments regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments (i.e., bone mass, fat mass, and fat-free/soft tissue mass). Quality control (QC) calibration procedures will be performed on a spine phantom (Hologic X-CALIBER Model DPA/QDR-1 anthropometric spine phantom) prior to each testing

session. In addition, weekly calibration procedures will be performed on a density step calibration phantom.

Resistance Exercise Machines. Maximum strength (1-RM strength) tests will be performed on the bench press, seated row, overhead shoulder press, and knee extension machines (Body Masters, Inc., Rayne, LA). Equipment and testing will be contained within the EBNL.

Muscle Biopsy Needle. The muscle biopsy technique will be performed with a 5-mm Bergstrom biopsy needle (shown in photos above).

Serum and Muscle Protein Analyses. Blood and muscle samples will also be used to assess hormone profiles spectrophotometrically using enzyme-linked immunoabsorbent assays (ELISA) with a Wallac Victor-1420 micoplate reader Wallac Victor-1420 micoplate reader (Perkin-Elmer Life Sciences, Boston, MA). The assays will be performed at either 405 or 450 nm wavlength against a known standard curve.

Participants

Recruitment

Ten apparently healthy, non-resistance trained [no regular, consistent resistance training (i.e. thrice weekly) for at least one year prior to the onset of the study], men between the ages of 18-30 will volunteer to participate in the study. Enrollment will be open to men of all ethnicities. A recruitment flyer that will be posted on campus and at area fitness centers is attached.

Selection Criteria

Participants will not be allowed to participate in the study if they:

1. have been involved in a habitual resistance training program (minimum of 3 hours/week for at least 1 year);
2. use tobacco products;
3. have orthopedic limitations that would limit participation in resistance training;
4. have a known allergy to topical anesthetics;
5. have any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism;
6. have a bleeding disorder, history of pulmonary disease, hypertension, hepatorenal disease, musculoskeletal disorders, neuromuscular/neurological diseases, autoimmune disease, cancer, peptic ulcers, anemia, or chronic infection (e.g., HIV);

7. are taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (e.g,thyroid, insulin, etc), emotional/psychotropic (e.g., Prednisone, Ritalin, Adderall), neuromuscular/neurological, or androgenic medications (anabolic steroids);
8. have taken ergogenic levels of nutritional supplements that may affect muscle mass (e.g., creatine, HMB) or anabolic/catabolic hormone levels (e.g., androstenedione, DHEA, etc) within three months prior to the start of the study.
9. have any absolute or relative contraindication for exercise testing or prescription as outlined by the American College of Sports Medicine;
10. report any unusual adverse events associated with this study that in consultation with Darryn Willoughby, Ph.D. who may recommend removal from the study.

Compensation or Incentives

Participants completing all familiarization and testing sessions as well as turning in all required materials (i.e., dietary logs) in the study will be paid $100. Participants may receive information regarding results of these tests if they desire. If subjects are Baylor students, they will not receive any academic credit for participating in this study.

Potential Risks

Participants who meet eligibility criteria will be exposed to a very low level of electrical current that will be passed through each subject's body using a bioelectrical impedance analyzer. This analyzer is commercially available and has been used in the health care/fitness industry as a means to assess body composition and body water for over 20 years. The use of the BIA analyzer has been shown to be safe methods of assessing body composition and total body water and is approved by the FDA.

Participants who meet eligibility criteria will be subjected to strength testing sessions involving dynamic muscle contractions. Participants in this study will not be experienced resistance trainers, and will be instructed to only perform the prescribed resistance training protocol throughout the duration of the study. As a result of the exercise protocol, participants will most likely experience short-term muscle fatigue. In addition, they will likely experience muscle soreness in muscles in the upper- and lower-body for up to 24 to 48 hours after exercise. This soreness is normal and should be commensurate with the type of muscle soreness participants may have felt after doing unaccustomed physical activity. Muscle strains/pulls resulting from 1-RM testing and the dynamic exercise protocol are possible. During the familiarization session, participants will be informed of the resistance training program and correct lifting technique for each exercise demonstrated. Therefore, potential injury due to exercise will be minimized since all participants will be instructed on how to adhere to correct lifting technique. In addition, only Darryn Willoughby, Ph.D., Pete Grandjean, Ph.D., Brian Leutholtz, Mike Spillane, M.S.Ed, or Neil Schwarz, M.S. will conduct the testing and exercise procedures. Participants will be made aware of the intensity and duration of the expected soreness due

to the exercise sessions. However, there are minor risks of muscular pain and soreness associated with the resistance training protocol required in this study which are not uncommon to any exercise program especially for individuals who do not resistance train on a regular basis. Participants will donate about approximately 20 milliliters of venous blood a total of 16 times during the study by way of an intravenous catheter and standard phlebotomy using sterile techniques by an experienced phlebotomist using standard procedures. These procedures may cause a small amount of pain when the needle is inserted into the vein as well as some bleeding and bruising. However, proper pressure will be applied upon removal to reduce bruising. The subject may also experience some dizziness, nausea, and/or faint if they are unaccustomed to having blood drawn.

Complications resulting from the muscle biopsy are rare, especially in this case where the biopsy is similar to receiving a routine intramuscular injection. As with the blood draw, however, there is a risk of infection if the subject does not adequately cleanse the area for approximately 48-72 hours post biopsy. While leaving the butterfly bandage in place, participants will be instructed to cleanse the biopsy area with soap and water every 4-6 hours, pat the area dry and reapply a fresh adhesive bandage. The participant will be instructed to leave the bandages on for 24 hours (unless unexpected bleeding or pain occurs) and asked to report back to the lab within 24 hours to have the old bandages removed, the incision inspected and new bandages applied. The participant will be further advised to refrain from vigorous physical activity with the affected leg for 24 hours after the biopsy. There is a potential risk of an allergic reaction to the Lidocaine. All subjects will be asked if they have known allergies to local anesthetics (e.g. Lidocaine, Xylocaine, etc.) that they may have been previously given during dental or hospital visits. Participants with known allergies to anesthesia medications will not be allowed to participate in the study. Darryn Willoughby, Ph.D. or Mike Spillane, M.S.Ed. will perform all muscle biopsies. Researchers involved in collecting data represent trained, non-physician, exercise specialists. All personnel involved in collecting data will be certified in CPR, which is also a condition to holding these professional certifications. A telephone and automated electronic defibrillator (AED) is located in the laboratory in case of any emergencies and there will be no less than two researchers working with each subject during testing. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance.

Potential Benefits

The main benefit that participants may obtain from this study is how the body produces testosterone in response to resistance exercise. In addition, participants may gain insight into how they can enhance muscle mass and performance that typically occurs in conjunction with resistance training as well as improved health profiles. Participants may also gain insight about their health and fitness status from the assessments to be performed.

Assessment of Risk

The greatest risk associated with participating in this study will likely be from the muscle soreness participants will experience from participating in the resistance exercise protocol. However, the intensity of the exercise protocol will be no more than when individuals engaged heavily in a new or different form of physical activity. Therefore, the potential benefits of subjects participating in this study outweigh the potential risks.

Compensation for Illness or Injury

Each participant will agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss suffered as a result of participation in this study regardless of the cause of injury, damage, or loss.

Confidentiality

Information obtained from this research (including questionnaires, medical history, laboratory findings, or physical examination) will be kept confidential to the extent permitted by law. However, according to FDA regulations, records will be open to FDA representatives to review if necessary. This may include questionnaires, medical history, laboratory findings/reports, statistical data, and/or notes taken throughout this study. Records of the research may also be subpoenaed by court order or may be inspected by federal regulatory authorities. Data derived from this study may be used in reports, presentations and publications. Participants in this study will not be individually identified unless they give their written consent. All participants will have a number to identify their results. Only the study personnel will know the subject numbers. Only study personnel will have access to the data. All data will be stored in a locked cabinet in the Exercise and Biochemistry Laboratory and only Darryn Willoughby, Ph.D. will have access to the key. Additionally, that confidentiality will be maintained by assigning code numbers to the files, limiting access to data to research assistants, locking cabinets that store data, and providing passwords to limit access to computer files to authorized personnel only. All evidence of primary data will be stored for exactly three years after the completion of the study. At this time data will be destroyed in a manner that instills complete privacy to all participants of the study. Analyzed muscle and blood samples will be discarded in an appropriately-labeled biohazard waste disposal container. However, unused muscle and blood samples will be kept in a locked freezer for no longer than one

year. If any subsequent analysis occurs with the samples, they will be re-coded to further instill confidentiality.

Data Presentation & Publication

Data will be presented at an appropriate scientific conference (e.g., American College of Sports Medicine, Experimental Biology, etc.) and published in a peer reviewed scientific journal (e.g., Medicine & Science in Sport and Exercise, Journal of Applied Physiology, etc.).

Statement on Conflict of Interest

Funding for this study will be provided by the Exercise and Biochemical Nutrition Laboratory of Baylor University. Researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Table 1. Overview of Research Design

Visit 1 (Familiarization and Entry)	Testing Session 1 (Visit 2)	24 Hour Follow-Up (Visit 3)	Testing Session 2 (Visit 4)	24 Hour Follow-Up (Visit 5)
Explanation of Study Procedures Demographic and Health History Form Activity Form General Exam to Determine Qualifications to Participate in Study. Informed Consent Form. Determination of Height and Body Weight. Determination of Resting Heart Rate and Blood Pressure Body Composition Assessment. Muscle Strength Assessments	Heart Rate and Blood Pressure Blood Collection Muscle Biopsy Diet Log Analysis Resistance Exercise Session	Heart Rate and Blood Pressure Blood Collection Muscle Biopsy	Heart Rate and Blood Pressure Blood Collection Muscle Biopsy Diet Log Analysis Resistance Exercise Session	Heart Rate and Blood Pressure Blood Collection Muscle Biopsy

BAYLOR UNIVERSITY

Department of Health, Human Performance, & Recreation

Informed Consent Form

Title of Investigation: Effects of Endogenous Elevations in Testosterone on Resistance Exercise-Induced Skeletal Muscle Androgen Receptor –Mediated Signaling and DNA Binding in Men

Principal Investigator: Darryn S. Willoughby, Ph.D.
Department of HHPR, Baylor University

Co-investigators: Pete Grandjean, Ph.D.
Department of HHPR, Baylor University

Brian Leutholtz, Ph.D.
Department of HHPR, Baylor University

Mike Spillane, M.S.Ed.
Department of HHPR, Baylor University

Neil Schwarz, M.S.Ed.
Department of HHPR, Baylor University

Sponsors: Exercise and Biochemical Nutrition Lab (Baylor University)

Rationale:

Resistance exercise stimulates skeletal muscle growth which results from protein accumulation within muscle fibers, thus promoting muscle fiber growth. There are a number of physiological mechanisms known to contribute to this process such as intrinsic factors within skeletal muscle, immune/inflammatory, and endocrine (hormonal). Of the endocrine factors, a number of studies have shown the ability of resistance exercise to increase the endogenous level of testosterone. Testosterone is an androgenic hormone with robust growth-promoting capabilities in skeletal muscle due to its ability to increase muscle protein synthesis. Being a fat-soluble hormone, testosterone will bind with its androgen receptor within skeletal muscle. Upon binding, this hormone-receptor complex migrates into the nucleus where it binds to a specific sequence within various muscle-specific genes, thereby enhancing gene expression. Resistance exercise affects androgen signaling via several mechanisms. First, resistance exercise elevates endogenous testosterone concentrations. Elevations in testosterone due to resistance exercise are known to increase muscle strength following long-term training. Second, muscle contraction increases muscle androgen receptor content due to enhancing androgen

receptor gene expression. Third, evidence indicates that elevations in endogenous testosterone following resistance exercise could feasibly increase muscle androgen receptor content independently of the influence of muscle contraction. This process of androgen signaling represents an important target for research aimed at promoting muscle growth and/or counteracting the muscle atrophy characteristic of aging, inactivity/immobilization, and various diseases. Therefore, the purpose of this study is to determine the influence of elevated endogenous circulating testosterone levels on skeletal muscle testosterone, androgen receptor protein, and myofibrillar protein concentration, as well as the mRNA expression of the androgen receptor.

Description of the Study:

I will be one of 10 apparently healthy, non-resistance-trained males between the ages 18 to 30 who will participate in this study. I understand that I will be required to visit the laboratory five times during the course of the study in the following manner: visit 1 = entry/familiarization session, visit 2 = testing/resistance exercise session 1, visit 3 = 24 hour follow-up for session 1, visit 4 = testing/resistance exercise session 2, visit 5 = 24 hour follow-up for session 2. Relative to the 2 testing sessions, I will perform a resistance exercise session involving the knee extension exercise on two occasions separated by three weeks. One session will constitute the control session and be preceded by rest and the other will constitute the experimental session and preceded by a bout of high-volume, moderate-intensity upper-body resistance exercise using short rest periods. At each of the 5 visits, I will have my heart rate and blood pressure assessed. At visits 2 – 5, I will also have blood and muscle samples obtained.

During an initial familiarization session (visit 1), I will be informed of the requirements of the study and sign an informed consent statement in compliance with the Human Subjects Guidelines of Baylor University and the American College of Sports Medicine. A trained individual will examine me to determine if I am qualified to participate in this study. If I am cleared to participate in the study, I will be familiarized to the testing procedures and will then undergo assessments for body composition and muscle strength. This session will take approximately 60 minutes to complete. Once I complete the familiarization session, I will be scheduled for the first resistance exercise session (visit 2).

During the familiarization session, I understand that I will have my maximum muscle strength determined on the bench press, seated row, overhead shoulder press and knee extension exercises. I will warm up by completing 5 to 10 repetitions with a very light weight and then complete 3 to 5 repetitions with a heavier weight. The weight will then be increased conservatively, and I will attempt to lift the weight for one repetition. If the lift is successful, I will rest for 2 minutes before attempting the next weight increment. This procedure will be continued until I fail to complete the lift. My maximum strength will be recorded as the maximum weight I am able to lift for one repetition. In addition, I will have my body composition (body fat and muscle mass) determined using dual-energy x-ray absorptiometry (DEXA). The DEXA body composition test will

involve me lying down on my back in a comfortable position in a pair of shorts/t-shirt. A low dose of radiation will then scan my entire body for approximately 6 minutes. Radiation exposure from the DEXA is approximately 1.5 mR per scan. This is similar to the amount of natural background radiation I would receive in one month while living in Waco, TX. The maximal permissible x-ray dose for non-occupational exposure is 500 mR per year. Total radiation dose will be less than 5 mR for the entire study.

Following the familiarization session, I will be instructed to refrain from exercise for 48 hours and fast for 8 hours prior to each resistance exercise session. I will be provided with a dietary analysis form that I am to complete for 4 days prior to each resistance exercise testing session. Once I report to the lab for each testing session, I will turn in my dietary analysis form.

I understand that I will be required to participate in a 2 separate testing sessions (visits 2 & 4) consisting of the knee extension exercise where I will perform 5 sets of 5 repetitions at 90% - 95% of my maximum strength. However, for one session I will perform 4 sets of 10 repetitions using 80% of my maximum strength on the bench press, seated row, and overhead shoulder press exercises immediately before the knee extension exercise.

During the study, I understand that I will donate about 20 milliliters (4 teaspoons) of venous blood from a vein in my arm by way of an intravenous catheter inserted into my arm using sterile techniques by an experienced phlebotomist using standard procedures. This procedure may cause a small amount of pain when the needle is inserted into the vein as well as some bleeding and bruising, and will remain in place until the end of the testing process. However, proper pressure will be applied upon removal to reduce bruising. I understand that I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn. I understand that personnel who will be inserting the intravenous catheter and taking my blood are experienced in phlebotomy (procedures to take blood samples) and are qualified to do so under guidelines established by the Texas Department of Health and Human Services. The process of inserting the catheter and blood draws at each sampling point will take about 5 minutes and I will be required to leave the catheter in place for the duration of the testing session. Throughout the testing, the catheter will be securely placed in my arm; however, during the 3 hours following resistance exercise I may read or participate in other sedentary activities. At visits 2 and 4 (resistance exercise/testing sessions 1 and 2) I will have blood obtained through the catheter at 8 different time points. However, 24 hours after each session (visits 3 and 5) I will have blood sampled through a standard venipuncture.

In addition to the blood draws, I will undergo the muscle biopsy. I understand that I will have the biopsy location identified on the thigh (opposite thigh for the second exercise session). The biopsy area will be shaved clean of leg hair, washed with antiseptic soap and cleaned with rubbing alcohol. In addition, the biopsy site will be further cleansed by swabbing the area with Betadine (fluid antiseptic). I understand that a small area of the

cleaned skin approximately 2 cm in diameter will be anesthetized with a 1.0 mL subcutaneous injection of the topical anesthetic Lidocaine. Once the local anesthesia has taken effect (approximately 2-3 minutes) the biopsy procedure will only take 15-20 seconds. Once anesthetized, I understand that a scalpel point will be used to make an incision approximately 1 cm in length through the skin. Due to the localized effects of the anesthetic, however, I should feel no pain during this process. At this point, I understand that the biopsy needle will be advanced into the incision approximately 1 cm and during this part of the procedure I may feel pressure in my thigh area. Once the muscle sample has been obtained, pressure will be immediately applied to the incision. With the incision being so small bleeding is slight; therefore, only a butterfly bandage is needed to close the incision, which will then be covered with a pressure bandage. I understand that I will be provided verbal and written instructions for post-biopsy care. I understand that I will be instructed to leave the butterfly bandage in place for 72 hours. However, I understand that I will be asked to report back to the lab 24 hours after the biopsy (unless unexpected bleeding or pain occurs) to have the old bandage removed, the incision inspected and new bandages applied, and that I will also report back to the lab for the same reason at 48 hours after the biopsy. I will be further advised to refrain from vigorous physical activity with my leg during the first 24 hours post-biopsy. I understand that if I feel it necessary I may take a non-prescription analgesic medication such as acetominophen to relieve pain if needed and that some soreness of the area may occur for about 24 hours after the biopsy. I will also be advised to avoid such medications such as aspirin, Advil, Bufferin, Nuprin, or Ibuprofen as they may lead to bruising at the biopsy site. I understand that I will be asked to undergo the muscle biopsy procedure on 8 separate occasions throughout the study.

I understand that when I report to the laboratory for the two testing/resistance exercise sessions on visits 2 and 4, I will turn in my 4-day dietary records. In addition, I will have my heart rate and blood pressure determined, and will also provide blood and muscle samples. I understand that if clinically significant side effects are reported from my participation in the study, I will be referred to discuss the problem with Darryn Willoughby, Ph.D. Upon his discretion, I may be referred to discuss the matter with my primary care physician to determine whether any medical treatment is needed and/or whether I can continue in the study. I understand that if I fail to report my progress and health status to the research assistant I may be removed from the study.

I agree to do my best to: 1) follow the instructions outline by the investigators; 2) show up to all scheduled testing times; and 3) put forth my best effort as instructed. I agree not to take any other nutritional supplements or performance enhancing aids during this study (i.e. vitamins/minerals, creatine, HMB, androstenedione, DHEA, etc). In addition, I agree not to take any non-medically prescribed medications and to report any medication that is prescribed for me to take during this study. I understand that if I take any

nutritional supplements or medications during the course of the study that I will be removed from the study.

Exclusionary Criteria

I understand that in order to participate in the study, a trained individual will examine me to determine whether I qualify to participate. I understand that I will not be allowed to participate in this study if: 1.) I have any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism; 2.) I have a history of pulmonary disease, hypertension, liver or kidney disease, musculoskeletal disorders, neuromuscular or neurological diseases, autoimmune disease, cancer, peptic ulcers, or anemia; 3.) I am taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (ie, thyroid, insulin, etc), psychotropic, neuromuscular/neurological, or androgenic medications; 4.) I have any bleeding disorders; 5.) I have any chronic infections (e.g., HIV); 6) I have a known allergic reaction to topical anesthetics.
I have reported all nutritional supplements, medically prescribed drugs, and non-medically prescribed drugs that I am presently taking. I have reported whether I have had any prior allergic reactions to topical anesthetics. I have completed medical history questionnaires and am not aware of any additional medical problems that would prevent me from participating in this study. I agree to report all changes in medical status, nutritional and/or pharmacological agents (drugs) that I take during the course of the investigation to Darryn Willoughby, Ph.D. (254-710-3504). I understand that if I experience any unexpected problems or adverse events from participating in this study I may be referred to discuss the problem with my primary care physician to determine whether any medical treatment is needed and/or whether I can continue in the study.

Risks and Benefits

I understand that there are minor risks of muscular pain and soreness associated with the resistance training protocol required in this study which are not uncommon to any exercise program especially for individuals who do not resistance train on a regular basis. On 16 separate occasions during this study, I understand that I will have approximately 4 teaspoons (20 milliliters) of blood drawn from a vein in my forearm. On 14 of these occasions, blood will be obtained using an in-dwelling venous catheter, whereas on 2 other occasions it will involve a standard blood draw using a sterile needle. All blood sampling will be performed by an experienced phlebotomist following an 8-hour fast. This procedure may cause a small amount of pain when the needle is inserted into my vein as well as some bleeding and bruising. I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn.

On 8 separate occasions during this study (4 at each testing/exercise session), I understand that I will undergo a muscle biopsy in which a small sample of muscle will be obtained from the thigh of my exercised leg. I understand that Darryn Willoughby, Ph.D. or Mike Spillane, M.S.Ed. will perform all of the biopsies and that a local anesthetic (Lidocaine) will be injected into the skin of my thigh prior to the biopsy, which will help

prevent any pain and discomfort during the procedure. I understand that I will have a small incision made in my skin and a biopsy needle introduced 1 cm into the incision. I also understand that the incision is so small that it will not require any stitches and will be simply closed with a butterfly bandage and then covered with an adhesive bandage (band-aid). After the anesthetic wears off within 3-4 hours, I understand that the sensation at the biopsy site is comparable to that of a bruise and may persist for 24-36 hours after the procedure. I understand that I am required to inform the study investigators if I have had any prior allergic reactions to anesthesia (e.g. while in the hospital or during a dental visit).

Alternative Treatments

This is not a medical treatment. Therefore, if medical treatment is needed, I must obtain treatment for any medical problem I might have from my personal physician.

Costs and Payments

If I am a Baylor University student, I will not receive any academic credit for participating in this study. I understand that if I am an intercollegiate scholarship athlete I may not be eligible to receive payment to participate in this study. Eligible participants will be paid $100 for completing the familiarization and experimental testing sessions. I also understand that I will be given free blood assessments during the course of the study as described above and may receive information regarding results of these tests if I desire.

New Information

Any new information obtained during the course of this research that may affect my willingness to continue participation in this study will be provided to me. In addition, I will be informed of any unusual/abnormal clinical findings in which medical referral to my personal physician may be warranted. If I desire, I may request that this information be provided to my physician.

Confidentiality

I understand that any information obtained about me in this research, including medical history, laboratory findings, or physical examination will be kept confidential to the extent permitted by law. However, I understand in order to ensure that FDA regulations are being followed, it may be necessary for a representative of the FDA to review my records from this study which may include medical history, laboratory findings/reports, statistical data, and/or notes taken about my participation in this study. In addition, I understand that my records of this research may be subpoenaed by court order or may be inspected by federal regulatory authorities. I understand that data derived may be used in reports, presentations, and publications. However, I will not be individually identified unless my consent is granted in writing. Additionally, that confidentiality will be

maintained by assigning code numbers to my files, limiting access to data to research assistants, locking cabinets that store data, and providing passwords to limit access to computer files to authorized personnel only. I understand that once blood and muscle samples are analyzed that they will be discarded.

Right to Withdrawal

I understand that I am not required to participate in this study and I am free to refuse to participate or to withdraw from the study at any time. Further, that my decision to withdraw from the study will not affect my care at this institution or cause a loss of benefits to which I might be otherwise entitled. If there is concern about my medical safety, I may be referred to seek medical attention.

Compensation for Illness or Injury

I understand that if I am injured as a direct result of taking part in this study, I should consult my personal physician to obtain treatment. I understand that the cost associated with the care and treatment of such injury will be the responsibility of me or my insurance carrier. In some cases, insurers may not reimburse claims submitted for a research-related injury resulting from medical procedures or treatments performed as part of a research study. I understand that Baylor University, the investigator's institutions, and the grant sponsor have not budgeted funds to compensate me for injury or illness that may result from my participation in this study and thus will not be accountable for illness or injury acquired during the course of this study. However, I may be referred to my personal physician if any clinically significant medical/psychological findings are observed during the course of this study.

I agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss I suffer as a result of my participation in this study regardless of the cause of my injury, damage or loss.

Statement on Conflict of Interest

I understand that this study is funded by the Exercise and Biochemical Nutrition Laboratory at Baylor University, and that the researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Voluntary Consent

I certify that I have read this consent form or it has been read to me and that I understand the contents and that any questions that I have pertaining to the research have been, or will be answered by Darryn Willoughby, Ph.D. (principal investigator, Department of Health, Human Performance & Recreation, 120 Marrs McLean Gymnasium, Baylor University, phone: 254-710-3504) or one of the research associates. My signature below

means that I am at least 18 years of age and that I freely agree to participate in this investigation. I understand that I will be given a copy of this consent form for my records. If I have any questions regarding my rights as a research subject in this study, I may contact Baylor's University Committee for Protection of Human Subjects in Research. The Chairman is Dr. Michael Sherr, School of Social Work, P.O. Box 97320, Waco, Texas 76798, phone number (254) 710-4483.

Date _____ Subject's Signature _____

I certify that I have explained to the above individual the nature and purpose of the potential benefits and possible risks associated with participation in this study. I have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

Date _____ Investigator's Signature _____

Part 5: Informed Consent Form Checklist

When using humans as subjects in research you must obtain their informed consent. Check each of the following items as they appear on your Informed Consent Form and include this checklist with your protocol:

__x_(a) A statement explaining the purpose of the research.

__x_(b) A statement of the expected duration of the subject's participation.

__x_(c) A description of the procedures to be followed.

__x_(d) A description of any reasonable foreseeable risks or discomforts to the subject, including invasion of privacy.

__x_(e) A description of any benefits resulting from the research, either to the subject or to others.

__x_(f) A statement that informs subject of his/her right not to be a subject in a research project that is also a teaching exercise.

__x_(g) A statement informing subject about how his/her anonymity will be guarded; i.e., that their confidentiality will be protected by assigned code numbers, by limitations of who has access to data, by data storage in locked cabinets, by locked computer files, etc.

__x_(h) A statement that the subject's participation is voluntary, and that his/her refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and that the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

_na_(i) A disclaimer, if applicable, regarding the use of the Internet to collect data.

__x_(j) For research involving more than minimal risk, an explanation regarding the availability of any compensation or any medical treatments if injury occurs (if applicable, see OHRP Reports).

__x_(k) If written informed consent is required, a place for the subject to sign and date the form and a statement that a copy of the signed consent form will be given to the subject for his/her records.

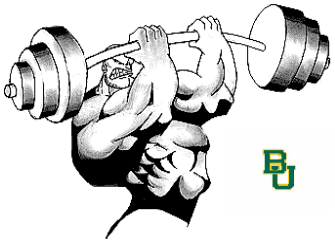
_na_(l) If the subject is a minor, a statement of parental responsibility in consenting to the child's participation in the study with a place for the parent to sign and date the form in addition to the participant's signature.

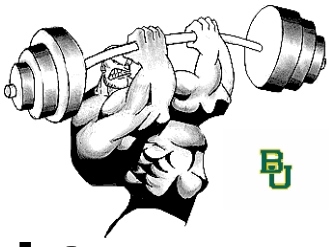
__x_(m) Include a short summary of your expertise related to this research proposal.

__x_(n) The name, address, and telephone number of the principal investigator of the research project, and his/her affiliation with Baylor University. If the principal investigator is a graduate student, the name and telephone number of the faculty advisor is also required.

__x_(o) A statement informing subject that inquiries regarding his/her rights as a subject, or any other aspect of the research as it relates to his/her participation as a subject, can be directed to Baylor's University Committee for Protection of Human Subjects in Research. The chairman is Dr. Matt Stanford, Professor Psychology and Neuroscience, PO Box

APPENDIX C





Want to get Paid to Workout?

Resistance-Trained Men Needed for a Weight Lifting Study

Researchers in the Exercise & Biochemical Nutrition Lab at Baylor University are recruiting 10 healthy, ***non-resistance-trained*** *men between the ages of* ***18-30*** *to participate in a study designed to evaluate the effects of their own endogenous testosterone on the effects of the androgen receptor and markers of muscle protein synthesis capacity. Participants will be required to engage in 2 resistance exercise sessions separated by 3 weeks. Participants will be required to undergo strength and body composition testing, and to also submit to providing blood samples and muscle biopsies. Eligible subjects will receive* ***$100*** *for completing the study and free muscle strength and body fat testing.*

For more information contact:

Exercise & Biochemical Nutrition Lab
Department of HHPR
Rena Marrs McLean Gymnasium Room 120
254-710-4012
Mike_Spillane@baylor.edu

Exercise • Biochemistry • Nutrition • Health
BU EBNL
BAYLOR UNIVERSITY
Exercise & Biochemical Nutrition Laboratory

APPENDIX D

Wound Care for the Muscle Biopsy Procedure

Complications resulting from the muscle biopsy procedure are rare. Furthermore, after the procedure, you can reduce your risk of chance of infection by adhering to the following course of action for wound care:

For approximately 24 hours post biopsy

leave the bandage(s) on for 24 hours (unless unexpected bleeding or pain occurs, which should be immediately reported to the lab)

- lightly clean around the bandage(s) if necessary
- report back to the lab within 24 hours to have the old bandage(s) removed, the incision inspected and new bandages applied
- refrain from vigorous physical activity with the leg during the first 24 hours post-biopsy

After the 24-hour follow-up, (for approximately 72 hours post biopsy)

- leave the butterfly bandage in place
- adequately cleanse the area surrounding the bandage with soap and water every 4-6 hours, and pat the area dry
- reapply a fresh adhesive bandage

At approximately 72 hours post biopsy

- return to the lab
- allow the incision sight to be inspected, butterfly removed, and new bandages applied
- leave these bandages on for 24 hours (unless unexpected bleeding or pain occurs)
- return to normal hygiene practices unless complications arise

Possible pain & side effects

Soreness of the area comparable to that of a bruise will likely persist for 24 hours, and possibly even 36 hours, after the biopsy procedure. Following the procedures outlined above should significantly minimize pain and possible bleeding of the area. However, some subjects experience no significant pain post biopsy.

☺ If needed, the subject may take non-prescription analgesic medication such as Acetominophen to relieve pain if needed.

☹ Medications such as aspirin, Advil, Bufferin, Nuprin, and Ibuprofen are discouraged as they may lead to excess bruising at the biopsy site.

If any questions or complications arise please contact:
Darryn Willoughby, Ph.D. Room # 120 Marrs McLean Gym (254) 710-3504
Darryn_Willoughby@baylor.edu

APPENDIX E

BAYLOR UNIVERSITY

EBNL

Medical History Inventory

Directions. The purpose of this questionnaire is to enable the staff of the Exercise and Sport Sciences Laboratory to evaluate your health and fitness status. Please answer the following questions to the best of your knowledge. All information given is **CONFIDENTIAL** as described in the **Informed Consent Statement.**

Name:_____ Age: _____Date of Birth: _____

Name and Address of Your Physician:

_____

MEDICAL HISTORY

Do you have or have you ever had any of the following conditions? (Please write the date when you had the condition in blank).

___ Heart murmur, clicks, or other cardiac findings?
___ Frequent extra, skipped, or rapid heartbeats?
___ Chest Pain (with or without exertion)?
___ High cholesterol?
___ Diagnosed high blood pressure?
___ Heart attack or any cardiac surgery?
___ Leg cramps (during exercise)?
___ Chronic swollen ankles?
___ Varicose veins?
___ Frequent dizziness/fainting?
___ Muscle or joint problems?
___ High blood sugar/diabetes?
___ Thyroid Disease?
___ Low testosterone/hypogonadism?
___ Glaucoma?
___ Asthma/breathing difficulty?
___ Bronchitis/Chest Cold?
___ Melanoma/Skin Lesions?
___ Stroke or Blood Clots?
___ Emphysema/lung disease?
___ Epilepsy/seizures?
___ Rheumatic fever?
___ Scarlet fever?
___ Ulcers?
___ Pneumonia?
___ Anemias?
___ Liver or kidney disease?
___ Autoimmune disease?
___ Nerve disease?
___ Psychological Disorders?

Do you have or have you been diagnosed with any other medical condition not listed?

_____
_____
_____
_____

Please provide any additional comments/explanations of your current or past medical history.

_____

_____

Please list any recent surgery (i.e., type, dates etc.).

_____

_____

_____

List all prescribed/non-prescription medications and nutritional supplements you have taken in the last 3 months.

What was the date of your last complete medical exam?

Do you know of any medical problem that might make it dangerous or unwise for you to participate in this study (including strength and maximal exercise tests) ____ If yes, please explain:

Recommendation for Participation

____ No exclusion criteria presented. Subject is ***cleared*** to participate in the study.

____ Exclusion criteria is/are present. Subject is ***not cleared*** to participate in the study.

Signed: _____ Date: _____

APPENDIX F

Baylor University
Exercise and Sport Nutrition Laboratory

Personal Information

Name:
Address:
City: _____ State: _____ Zip Code _____SS# _____

Home Phone: (___) _____ Work Phone: (___)

Beeper: (___) _____Cellular (___) _____

Fax: (___) _____email address:_____

Birth date: _____ / _____ / _____ Age: _____ Height: _____ Weight:

Exercise & Supplement History/Activity Questionnaire

Describe your typical occupational activities.

Describe your typical recreational activities

Describe any exercise training that you routinely participate.

How many days per week do you exercise/participate in these activities?

How many hours per week do you train?

How long (years/months) have you been consistently training?

When was the last time you ingested any nutritional supplements?

What was the reason you were taking these supplements?

APPENDIX G

Baylor University

Exercise & Sport Nutrition Laboratory

NAME _____ Date _____

INSTRUCTIONS

1. Record everything you eat for 4 days (including one weekend day). If you eat pretzels, record how many. If you eat a bag of chips, record the number of ounces. For drinks, record the number of cups or ounces. Record everything you drink except water.
2. Record the Food, Amount, Brand Name, and Preparation Methods. For example: baked vs. fried chicken; 1 cup of rice; 2 teaspoons of margarine; 1 cup of 2% milk; McDonald's, Healthy Choice, or Frosted Flakes.
3. Record immediately after eating. Waiting until that night may make it difficult to remember all foods and quantities.

Food (include brand)	Method of Preparation	Quantity (cups, oz., no.)
BREAKFAST:		
LUNCH:		
DINNER:		
SNACKS:		

APPENDIX H

Baylor University: Exercise & Biochemical Nutrition Laboratory
Trial: Endogenous Testosterone – Resistance Exercise

Demographics Staff Initials: _____

Name: Testing Session:

Date: _____ D.O.B.: Age: ___

Physiological Parameters: Fasted (8hrs)_____ URINE gravity _____ (< 1.02) BIA_____
Height 73.5 in Weight _____ Diet logs_____

PRE: **Time:_____**

1 Resting Heart Rate _____ BPM Blood Pressure _____/_____ Blood ____ Biopsy_____

Exercise:

Bench Press: _____ Weight

Set 1 - [10 reps] _____reps; _____ lbs *rest* 2 min

Set 2 - [10 reps] _____reps; _____ lbs *rest* 2 min

Set 3 – [10 reps] _____reps; _____ lbs *rest* 2 min

Set 4 – [10 reps] _____reps; _____ lbs *rest* 2 min

Seated Rows: _15_ Weight

Set 1 - [10 reps] _____reps; _____ lbs *rest* 2 min

Set 2 - [10 reps] _____reps; _____ lbs *rest* 2 min

Set 3 – [10 reps] _____reps; _____ lbs *rest* 2 min

Set 4 – [10 reps] _____reps; _____ lbs *rest* 2 min

Shoulder Press: _____ Weight

Set 1 - [10 reps] _____reps; _____ lbs *rest* 2 min

Set 2 - [10 reps] _____reps; _____ lbs *rest* 2 min

Set 3 – [10 reps] _____reps; _____ lbs *rest* 2 min

Set 4 – [10 reps] _____reps; _____ lbs *rest* 2 min

Knee Extensions: _____ Weight

Set 1 - [5 reps] _____R _____L *rest* 2 min

Set 2 - [5 reps] _____R _____L *rest* 2 min

Set 3 – [5 reps] _____R _____L *rest* 2 min

Set 4 – [5 reps] _____R _____L *rest* 2 min

Set 5 – [5 reps] _____R _____L **Time:_____**

POST: ----------10 min post----------

2 Heart Rate _____ BPM Blood Pressure _____/_____ Blood ____ Biopsy_____

30 Min: ---------- **Time:_____**----------

3 Heart Rate _____ BPM Blood Pressure _____/_____ Blood ____

1 hour: ----------

4 Heart Rate _____ BPM Blood Pressure _____/_____ Blood ____

2 hour: ----------

5 Heart Rate _____ BPM Blood Pressure _____/_____ Blood ____

3 hour: ----------

6 Heart Rate _____ BPM Blood Pressure _____/_____ Blood ____ Biopsy_____
Time:_____

Baylor University: Exercise & Biochemical Nutrition Laboratory
Trial: Endogenous Testosterone – Resistance Exercise

Demographics Staff Initials: _____

Name:_____ Testing Session Day 2

Date: _____ D.O.B.: _____ Age: _____

Physiological Parameters: Fasted (8hrs)_____ URINE gravity _____ (< 1.02)

24 hour post: **Time:_____**

7 Resting Heart Rate _____ BPM Blood Pressure _____/_____ Blood ____ Biopsy_____

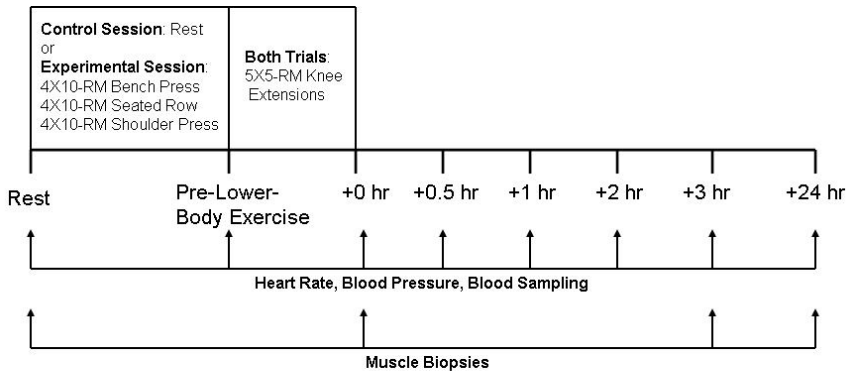


Figure 1. An illustration of the experimental protocol to be used in the study. At each of the 8 time points, heart rate and blood pressure will be assessed and blood samples will be obtained. Muscle biopsies, however, will be obtained at rest, +0 hr, +3 hr, and +24 hr.

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