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Effects of d-aspartic acid on testosterone and training outcomes in a resistance trained population: Findings from an acute dosing study, and a three-month training study.

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A thesis submitted for the degree of Doctor of Philosophy at the Western Sydney University in 2016

School of Science and Health

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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Geoffrey William Melville

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LIST OF ABBREVIATIONS

Abbreviation	Definition
1RM	one repetition maximum
α-MN	alpha motor neuron
Akt	protein kinase b-Akt pathway
ALB	albumin
AR	androgen receptor
AP	action potential
BT	basic training
cAMP	cyclic adenosine monophosphate
CSA	cross-sectional area
CV	coefficient of variation
DAA	d-aspartic acid
D0	placebo group in study 1
D3	three-gram group in study 1
D6	six-gram group in study 1
DDO	d-aspartic acid oxidase
E_2	estradiol
EDTA	ethylenediaminetetraacetic acid
EMG	electromyography
EFOV	extended-field-of-view
FT	free testosterone
GnRH	gonadotropin releasing hormone
HDL	high density lipoprotein
hCG	human chorionic gonadotropin
HPG	hypothalamic-pituitary-gonadal (axis)
H-reflex	Hoffman reflex

Ia	type Ia sensory fibre
IIa	type IIa muscle fibre type
iEMG	integrated electromyography
IGF-1	insulin like growth factor 1
IVF	in vitro fertilisation
LH	luteinizing hormone
MAV	mean average EMG
MEP	motor evoked potentials
M_{max}	maximal motor response
MN	motor neuron / motoneuron
MPB	muscle protein breakdown
MPS	muscle protein synthesis
mRNA	messenger ribonucleic acid
mTOR	mechanistic target of rapamycin
MVC	maximal voluntary contraction
NMDA	n-methyl-d-aspartic acid
NMA	n-methyl-d, l-aspartate
p70S6k / S6K1	ribosomal protein S6 kinase beta-1
PA	phosphatidic acid
PI3K	phosphatidylinositol 3-kinase
PLA	placebo group in study 2
PWR	power
RER	rate of EMG rise
RF	rectus femoris
RM	repetition maximum prescriptions
RT	resistance trained/training/trainer
RFD	rate of force development
sEMG	surface EMG
SHBG	sex-hormone-binding-globulin

StAR	steroidogenic acute regulatory protein
rpS6 / S6	ribosomal protein S6
SAMe	S-adenosyl-L-methionine or S-adenosyl methionine
SC	satellite cells
SST	serum separator tubes
T:C	testosterone/cortisol ratio
T:SH	testosterone/SHBG ratio
T1	baseline time point (study 1 and 2)
T2	beginning of experimental period (study 1), week 6 (study 2)
T3	post testing (study 1 and 2)
TMS	transcranial magnetic stimulation
TSC2	tuberous sclerosis complex 2 (tuberin)
TT	total testosterone
VI	vastus intermedialis
VL	vastus lateralis
VM	vastus medialis
V-wave	volitional wave

ABSTRACT

Previous research has demonstrated the effectiveness of the supplement d-aspartic acid to increase testosterone levels in infertile men. With this supplement readily available on the market, supplement companies are capitalising on the purported benefits to improve the resistance training goals of strength and hypertrophy. There is a requirement for more research to fully elucidate these claims within the context of a resistance trained population, and investigate the proposed mechanisms of action. Thus the effectiveness of the supplement d-aspartic acid to improve resistance training gains in resistance-trained men was studied throughout this thesis. This was achieved by 1) observing changes in testosterone over a 2-week dosing study and 2) investigate any testosterone-mediated improvements in strength, power, hypertrophy and neural adaptation over a three-month training study. A secondary objective was to clarify if any relationship exists between gonadal hormones and training related outcomes. Study 1 investigated three dosages: placebo, three grams and six grams of d-aspartic acid, taken daily throughout a two-week combined supplementation and training protocol. No changes in hormones were detected in the three-gram group. Participants within the six-gram group experienced significant reductions in both total testosterone and free testosterone. This reduction suggested that the higher dosage of d-aspartic acid was affecting negative feedback mechanisms within the hypothalamic-pituitary-gonadal axis or potentially over accumulating within the testes, causing disruption to testosterone production. As this was the first study to implement a higher dose of six grams per day, it was unknown what may occur if this dosage was consumed over a longer timeframe. It was important to explore the effects of this dosage further within the context of a long-term training study. Study 2 implemented a 12-week periodised training protocol combined with six grams daily d-aspartic acid supplementation. The

d-aspartic group experienced a reduction in estradiol, with no observed change in testosterone. Additionally, supplementation caused an increase in the early rate of force development within the quadriceps muscle. Peripheral neural adaptation of the gastrocnemius muscle was observed in the placebo group, but not in the d-aspartic group. Both groups increased hypertrophy and, strength within the quadriceps and calf muscles to a similar extent. No relationships were observed between testosterone change and hypertrophy or isometric strength change. A positive relationship was observed in change scores between estradiol and h-reflex recruitment gain of the soleus and gastrocnemius muscles. A negative relationship was observed between estradiol and the neural excitability of the soleus muscle, at the level of the motoneuron. Both relationships suggested that reduced levels of estradiol may be negatively impacting spinal plasticity within the calf muscles. The long-term supplementation of d-aspartic, even at the higher dosage of six grams, had no positive influence on testosterone levels in resistance trained men. The reduction observed in estradiol, while not affecting strength or hypertrophy, may have disrupted improvement in calf spinal excitability within this population.

This thesis supports and expands upon the current d-aspartic acid literature in resistance trained men. Results suggest that supplementation is ineffective at increasing testosterone levels, and has no ability to yield greater improvements in strength or hypertrophy from 12 weeks of resistance training. D-aspartic acid appears to have some interesting effects on neural adaptation and rate of force development that warrant further investigation to determine if it is a viable supplement for the improvement of quadriceps power.

CHAPTER 1 – INTRODUCTION

Thesis Statement

In the strength and conditioning community a key determinant, with regards to training gains, is the anabolic hormone testosterone. Circulating testosterone both directly imparts anabolic action, by interaction with androgen receptors (Heemers & Tindall, 2007) and indirectly, via increases in other growth factors, such as insulin-like-growth-factor 1 (Wu et al., 2007). Interaction with androgen receptors increases satellite cell activity and proliferation, causing a cascade of events leading to protein synthesis and muscle growth (Heemers & Tindall, 2007). Positive correlations between testosterone levels and training related strength gains have been observed in resistance trained populations (Häkkinen, Pakarinen, Alen, Kauhanen, & Komi, 1988b; Häkkinen, Pakarinen, Alen, & Komi, 1985). Resistance trained populations also tend to have higher basal testosterone levels (Häkkinen et al., 1988b; Häkkinen, Pakarinen, et al., 1985; Willoughby & Leutholtz, 2013; Willoughby, Spillane, & Schwarz, 2014), which may be an adaptive mechanism to improve strength and hypertrophy gains within this population. The importance of testosterone is also highlighted in anabolic steroid research, whereby the exogenous elevation of testosterone to supraphysiologic levels clearly has the ability to improve strength and hypertrophy, with and without a training stimulus (Bhasin et al., 1996). Due to these factors, the supplement industry has endeavoured to monetize various testosterone boosters. One recent testosterone booster which has entered the market is d-aspartic acid (DAA).

Aspartic acid ($C_4H_7NO_4$) is a α-amino acid which is known to exist in two isoforms, l-aspartic acid and d-aspartic acid. (2R)-2-aminobutanedioic acid or d-aspartic acid (DAA), previously believed to be exclusive to brain tissue in octopus, squid and cuttlefish, has also been shown to exist in mammals (A. D'Aniello et al., 1996). Free DAA is found in tissues and cells related to the central nervous and endocrine systems (A. D'Aniello, 2007; Furuchi & Homma, 2005). DAA is believed to stimulate the production and release of testosterone through multiple pathways of the hypothalamic-pituitary-gonadal (HPG) axis. It has been shown to increase steroidogenic acute regulatory protein (StAR) gene expression, in rat Leydig cells (Nagata, Homma, Matsumoto, & Imai, 1999). StAR is a key regulator for enhancing the transport of cholesterol, from the outer to the inner mitochondrial membrane (Furuchi & Homma, 2005). This transportation of cholesterol is believed to be the rate-limiting step in the production of steroid hormones, therefore by increasing levels of StAR, DAA may indirectly increase testosterone (Stocco, 1998). *In vitro* rat studies have demonstrated that DAA increases levels of testosterone, luteinizing hormone, progesterone (A. D'Aniello et al., 1996) and growth hormone (A. D'Aniello et al., 2000). This is believed to occur due to the accumulation of DAA in the anterior pituitary and testes (A. D'Aniello et al., 2000). Additional *in vitro* studies – on isolated rat testes (A. D'Aniello et al., 1996) and Leydig cells (Nagata, Homma, Lee, & Imai, 1999) – indicate that DAA increases the rate of testosterone synthesis in a dose-dependent manner. In these animals, the maximal-effective-dose of DAA, which elicited the greatest hormonal response (LH, testosterone and progesterone), was 1 μmol/g (A. D'Aniello et al., 1996). In humans, data partially supports the efficacy of DAA to increase testosterone, with positive improvements observed in infertile men consuming a 3 g/d dosage (D'Aniello et al., 2012; Topo, Soricelli, D'Aniello, Ronsini, & D'Aniello, 2009).

Currently, there is no information about the effect of different doses of d-aspartic acid in humans. Furthermore, the longest period this supplement has been researched in resistance trained men was over a period of one month, and in this study, three grams of d-aspartic acid did not increase basal testosterone (Willoughby & Leutholtz, 2013). It is possible that a larger dose is required in this population to increase basal testosterone. Elevated testosterone over the course of a training program could increase the chance of interaction with androgen receptor sites (Heemers & Tindall, 2007), upregulate satellite cell activity and increase levels of other anabolic hormones (Wu et al., 2007), leading to greater strength gains via hypertrophic adaptation. DAA may also work directly on mechanisms of neural adaptation, via its proposed role as a neurotransmitter (S. D'Aniello, Somorjai, Garcia-Fernàndez, Topo, & D'Aniello, 2011; Spinelli et al., 2006), leading to increased strength or power gains.

D-aspartic acid in resistance trained men

To date, three studies on pure d-aspartic acid supplementation (D'Aniello et al., 2012; Topo et al., 2009; Willoughby & Leutholtz, 2013), and two studies on DAA proprietary blends have been conducted in humans (Rodgers, Schriefer, Gunnels, & Bloomer, 2016; Willoughby et al., 2014). One proprietary blend study involved the methylated form of DAA (NMDA) (Willoughby et al., 2014). NMDA is believed to have similar hormonal effects as DAA (Arias et al., 1996; A. D'Aniello et al., 2000; Estienne, Hurlock, & Barb, 1998; Ondo, Wheeler, & Dom, 1988) and can also be synthesised from DAA (G. D'Aniello et al., 2000). Early research in humans showed promising increases in testosterone levels after short (Topo et al., 2009) and long term (D'Aniello et al., 2012) supplementation protocols in participants with fertility issues. After 12 days of supplementation (3.12 g/d), in a population of healthy males (27–37

years), levels of testosterone were significantly increased by 42% (4.5–6.4 ng/ml) (Topo et al., 2009). Recently it was reported that after 29 days of supplementation (3 g/d) and resistance training, levels of total testosterone and free testosterone were not significantly altered (Willoughby & Leutholtz, 2013). Training status and the accompanying basal testosterone levels may, in part explain the difference in outcome between these two studies. Topo et al. recruited sedentary male *in vitro* fertilisation (IVF) patients with low basal testosterone levels (~4.55 ng/ml) (Topo et al., 2009). In contrast, Willoughby & Leutholtz recruited resistance trained men who exhibited a higher average baseline testosterone count (~7.96 ng/ml) (Willoughby & Leutholtz, 2013). Resistance trained (RT) men tend to demonstrate higher levels of basal testosterone ranges than novice trainers. Basal testosterone levels of RT men range from approximately 5.1–12.0 ng/ml , (Häkkinen, Pakarinen, Alen, Kauhanen, & Komi, 1988a; Häkkinen, Pakarinen, et al., 1985; Willoughby et al., 2014) and novices range from about 3.7–8.5 ng/ml (Craig, Brown, & Everhart, 1989; Häkkinen et al., 2002; McCall, Byrnes, Fleck, Dickinson, & Kraemer, 1999; Reaburn, Logan, & Mackinnon, 1997; Staron et al., 1994). A larger dose for RT men may be needed to raise testosterone levels beyond this apparent adaptation to chronic resistance training.

Current research has only explored the effectiveness of one dosage of DAA – 3 g/d (Topo et al., 2009; Willoughby & Leutholtz, 2013) – hence the maximum effective dose for humans is yet to be determined. Supplement companies are currently recommending three grams of DAA once to twice a day, with these recommendations drawn from the 3 g/d literature. It is reasonable to believe that in RT males, a higher dose may be required to upregulate mechanisms of testosterone production such as enhanced cholesterol transport; an increase in testosterone regulatory hormones; and the accumulation of DAA within various glands along the HPG axis. This boost in

testosterone over time may equate to greater hypertrophy and neural adaptation, via an increased exposure of testosterone to androgen receptors located within muscle and nerve cells. These improvements may be further expressed by a gross increase in strength.

Hypertrophic adaptation

Resistance exercise training is well-known to lead to hypertrophy of skeletal muscle. However, the relative effect that basal testosterone change has on hypertrophy (within normal physiological ranges) is not abundantly clear. Hypertrophy is a result of an increase in the cross-sectional area (CSA) and the total volume of the muscle (Bruce, Phillips, & Woledge, 1997; Flann, Lastayo, McClain, Hazel, & Lindstedt, 2011). An important mechanism that attributes to hypertrophy is signalling pathways which initiate muscle protein synthesis (MPS). Testosterone is believed to be a major driver for the initiation of these signalling responses. Testosterone interacts with androgen receptors located on the muscle cell. Testosterone binds to the androgen receptor to up-regulate transcription rates and results in an increase in muscle protein synthesis (Heemers & Tindall, 2007). The importance of the testosterone-androgen receptor complex is supported by research where AR activity was blocked in rats. The rats that were exposed to an AR antagonist experienced significantly lower levels of muscle hypertrophy in comparison to control (Inoue, Yamasaki, Fushiki, Okada, & Sugimoto, 1994). Testosterone also stimulates the proliferation and differentiation of satellite cells (SC), which has been demonstrated in young (Sinha-Hikim, Roth, Lee, & Bhasin, 2003) and old (Sinha-Hikim, Cornford, Gaytan, Lee, & Bhasin, 2006) people, as well as increasing IGF-1 (Wu et al., 2007). Thus, an increase in testosterone levels,

mediated by DAA supplementation, could improve hypertrophy via the aforementioned mechanisms.

Neural adaptation

A fundamental mechanism for strength development is adaptation within the peripheral or central nervous systems. Androgen receptors have been found in a number of mammal nervous tissues including rat (Jordan, Price Jr, & Handa, 2002), gerbils (Mansouri, Siegford, & Ulibarri, 2003) and human fetal brain tissue (Hammond et al., 2001). It is believed that the AR may help grow and maintain the relative size of these motor neurons throughout normal life. In vitro experiments on human fetal brain tissue suggests that testosterone provides neuroprotective benefits (Hammond et al., 2001). It stands to reason that AR may exist within human motor neurons and that testosterone interaction could be beneficial to neuromuscular adaptation. Thus a boost in testosterone from DAA supplementation could increase strength via neural adaptation mechanisms.

One study to date has explored transitory increases in testosterone and changes with the corticospinal pathway in humans (Bonifazi, Ginanneschi, Della Volpe, & Rossi, 2004). Researchers injected 5000 IU of human chorionic gonadotropin (hCG) directly into participant's muscles, causing gonadal stimulation. This artificially increased testosterone levels, within normal clinical ranges (4.95 to 8.73 ng/ml). These increases were concurrent with reduced threshold of the evoked potentials from transcranial magnetic stimulation (TMS), indicating that testosterone can positively increase the output from a given input, within the corticospinal pathway. This suggests that artificially increasing testosterone levels, via supplementation may improve strength

or power, by increasing the efficiency of the corticospinal pathway. Adaptation of this pathway can be investigated by observing EMG responses to electrical nerve stimulation.

Spinal adaptation mechanisms can be investigated by observing changes in the Hoffman reflex (H-wave) (Aagaard, Simonsen, Andersen, Magnusson, & Dyhre-Poulsen, 2002b). Increases in H-waves have been noted as a result of resistance training (Aagaard et al., 2002b; Holtermann, Roeleveld, Engstrøm, & Sand, 2007; Lagerquist, Zehr, & Docherty, 2006), indicating adaptation of the Ia spinal reflex pathway. These papers collected H-wave data at a single stimulus intensity, however by collecting data along the entire ascending limb of the H-reflex curve, the change in excitability of motor units with various recruitment thresholds can be investigated (Vila-Cha, Falla, Correia, & Farina, 2012). The resistance training group experienced reductions in the stimulus intensity for H-wave threshold, suggesting excitability of lower threshold motor units. Conversely, the endurance training group observed reductions at high and low stimulus intensities, indicating increased excitability across the threshold spectrum (Vila-Cha et al., 2012). Supraspinal adaptation can be explored by observing changes in volitional waves (V-waves). V-waves are recorded during a maximal voluntary contraction (MVC) while simultaneously stimulating at a supramaximal intensity (130% of the maximal motor response - M_{max}). The V_{max}/M_{max} ratio is believed to be an indication of increased supraspinal drive (Aagaard et al., 2002b). Augmented supraspinal activation from the motor cortex increases transmission efficiency to the motor units and firing rate of the motor units, resulting in an increased force output (Aagaard et al., 2002b; Van Cutsem, Duchateau, & Hainaut, 1998). Research has previously demonstrated increased V-waves after strength training within novice populations (Aagaard et al., 2002b; Vila-Cha et al.,

2012). Currently within the literature, there is a lack of data exploring the changes in V-wave and H-reflex data in a resistance trained population. Furthermore, there is scarce data on the relationship between basal testosterone and neural adaptation. If d-aspartic acid mediated increases in testosterone effect strength via neural mechanisms, the combination of H-wave and V-wave data would help clarify if the adaptation is originating from supraspinal centres or along the reflex arc of the Ia afferents (Aagaard et al., 2002b; Vila-Cha et al., 2012).

Overview

The current literature indicates that supplementation of d-aspartic acid (3 g/d), has the potential to increase resting levels of testosterone in clinical populations, but not in resistance trained men. The first study of this thesis will measure testosterone levels within a resistance trained population, after 14 days of combined d-aspartic acid supplementation and resistance training. This research will examine whether 6 g/d of d-aspartic acid is more effective than 3 g/d and if so, by how much. The second study of this thesis will examine the long-term effects of d-aspartic acid on hormonal change, investigating any hormonally mediated effects on training outcomes, following a 12-week resistance training program. Training outcomes measured will include, changes in strength, hypertrophy and adaptation of nervous system. Consequently, this thesis will be based on original research that strives to study the effectiveness of d-aspartic acid, both for its potential to raise testosterone levels and the effect on training outcomes in resistance trained populations.

Aims objectives and hypotheses

Thesis aim

The primary aim of this thesis was to explore the effectiveness of the proposed testosterone boosting supplement: d-aspartic acid, in resistance trained men. This aim was achieved by 1) observing changes in testosterone over a 14-day dosing study and 2) implementing a three-month training study, noting changes in testosterone from supplementation, over a longer timeframe, as well as any testosterone-mediated improvements in strength, power and hypertrophy.

Objectives study 1

The objective of the first study was to evaluate the effects of two doses of d-aspartic acid (3g and 6g) on basal testosterone levels in resistance trained men. A secondary objective was to establish if a relationship exists between basal testosterone levels and responsiveness to DAA.

Hypotheses study 1

H_1: Testosterone levels will be unchanged in the three-gram group.

H_2: Testosterone levels will be increased in the six-gram group.

H_3: Lower initial testosterone levels would correspond with increased responsiveness to d-aspartic acid.

Objectives study 2

The primary objective of study two was to evaluate the long-term effectiveness of d-aspartic acid at a 6 g/d dosage, on basal testosterone levels during three months of resistance training. A secondary objective of this study was to reveal potential mechanisms of any training-related gains in strength, hypertrophy and power. The final objective was to clarify if any relationship exists between hormonal change and training-related improvements, in a resistance trained population.

Hypotheses study 2

H_1: Six grams of daily supplementation of d-aspartic acid during three months of training will result in increased basal testosterone as compared to placebo.

H_2: Six grams of daily supplementation of d-aspartic acid during three months of training will result in larger gains in isometric strength in the seated leg extension and calf raise as compared to placebo.

H_3: Six grams of daily supplementation of d-aspartic acid during three months of training will result in greater hypertrophy of the quadriceps and calf muscles as compared to placebo.

H_4: A positive relationship will be observed between testosterone levels and quadriceps hypertrophy.

H_5: A positive relationship will be observed between testosterone levels and isometric strength of the calf and quadriceps muscles.

CHAPTER 2 – LITERATURE REVIEW

The conceptual and empirical aspects of the research

The figure below depicts the conceptual framework for the study.

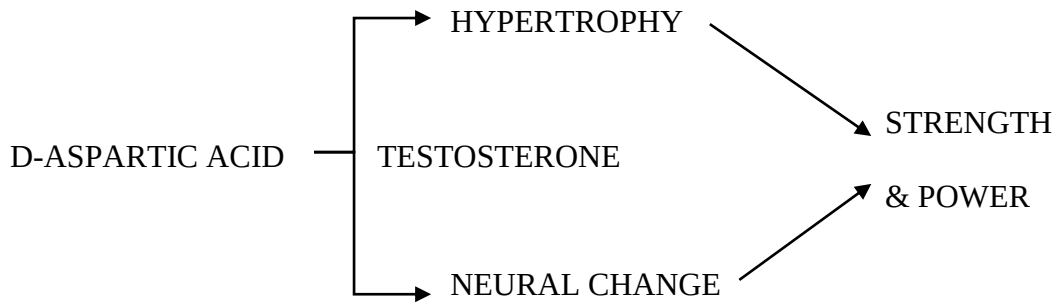


Figure 1 Conceptual Model

The conceptual model investigated in this thesis is that the relationship between d-aspartic acid supplementation (independent variable) and improvements in dynamic strength (dependent variable) will be moderated by elevated basal testosterone, with proposed mechanisms of action for the strength increase related to neural and hypertrophic adaptation.

D-Aspartic Acid

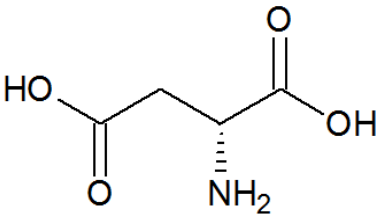


Figure 2 D-Aspartic Acid

Aspartic acid is a α-amino acid which is known to exist in two isoforms, l-aspartic acid and d-aspartic acid. (2R)-2-aminobutanedioic acid or d-aspartic acid (DAA), previously believed to be exclusive to brain tissue in octopus, squid and cuttlefish, has also been shown to exist in mammals (A. D'Aniello et al., 1996). Free DAA is found in tissues and cells related to the central nervous and endocrine systems (A. D'Aniello, 2007; Furuchi & Homma, 2005).

In rat's maximal levels of DAA production occur during the embryonic stage at the same time when testosterone production is greatest (A. D'Aniello et al., 1996). D-aspartic acid appears to stimulate testosterone production through multiple pathways. When rats are given DAA, it is shown to accumulate in the anterior pituitary and testes, which occurs in parallel with an increase in blood levels of luteinizing hormone, testosterone and progesterone (A. D'Aniello et al., 1996). *In vitro* studies on isolated testes indicate that DAA increases the rate of testosterone synthesis (A. D'Aniello et al., 1996). Research on purified rat Leydig cells demonstrates that cells cultured in DAA for 3+ hours increased production of testosterone in a dose-dependent manner (Nagata, Homma, Lee, et al., 1999). Additional *in vivo* rat studies has shown that 5 hours after injection DAA has a high affinity for accumulating in the anterior pituitary and moderate affinity for the testes, adrenals and hypothalamus (A. D'Aniello et al., 2000). The authors also reported that DAA supplementation caused an observed increase in growth hormone (GH), luteinizing hormone (LH), testosterone and progesterone. The combination of these experiments suggests DAA acts on the

aforementioned tissues to release the hormones, either directly or indirectly at various points on the hypothalamic–pituitary–gonadal axis (see Figure 4 on page 16). Alternatively, DAA may be synthesised into N-methyl-D-aspartic acid, which has similar properties to DAA.

N-methyl-D-aspartic acid (NMDA) or N-methyl-D,L-Aspartate (NMA), is the methylated forms of DAA and is believed to contribute to the mechanisms that underlie various hormone release in animal models. Evidence suggests that NMDA indirectly induces increased secretion of pituitary hormones, LH (Arias et al., 1996; Arslan, Pohl, & Plant, 1988; Bourguignon, Gerard, & Franchimont, 1989; A. D'Aniello et al., 2000; Estienne et al., 1998; Gay & Plant, 1987; Ondo et al., 1988; Pohl, Lee, & Smith, 1989; Price, Olney, Mitchell, Fuller, & Cicero, 1978; Schainker & Cicero, 1980; Wilson & Knobil, 1982), GH (Gay & Plant, 1987; Wilson & Knobil, 1982), (Chang, Barb, Kraeling, Rampacek, & Asanovich, 1993; A. D'Aniello et al., 2000; Downing, Joss, & Scaramuzzi, 1996; Estienne et al., 1996; Estienne et al., 1998; Estienne, Schillo, Green, Hileman, & Boling, 1989; Holloway & Leatherland, 1997; Pinilla, Gonzalez, Tena-Sempere, Dieguez, & Aguilar, 1999) and Prolactin (PRL) (Chang et al., 1993; Pohl et al., 1989). NMDA is believed to stimulate the release of these hormones via action at the hypothalamus – specifically the medial preoptic nucleus (Ondo et al., 1988) – releasing Gonadotropin-releasing hormone (GnRH) (Arias et al., 1996; Bourguignon et al., 1989; Zanisi & Messi, 1991). One study has shown that NMDA can also directly stimulate the rat pituitary gland (*in vitro* evidence of LH released from anterior pituitary) (Zanisi & Messi, 1991). It is understood that NMDA can be synthesised from DAA. With administration of DAA to Wistar male rats there is a significant increase in the concentration of NMDA (2h post injection) occurring in the hypothalamus (3.26-fold increase), anterior pituitary, hippocampus

(both ~2.8-3.0-fold increase), and to a lesser extent "total brain" and liver (G. D'Aniello et al., 2000). Additional experiments confirmed similar findings *in vitro* (G. D'Aniello et al., 2000). Tissue samples were incubated with DAA and S-adenosyl-L-methionine (SAMe) – again with the hypothalamus demonstrating to be the tissue where NMDA biosynthesis occurred at the highest rate – followed by the hippocampus, anterior pituitary, brain and liver (G. D'Aniello et al., 2000). When incubated with DAA and NMDA (A. D'Aniello et al., 2000), the hormonal release of GH was increased, which was enhanced in the anterior pituitary + hypothalamus model, as opposed to the anterior pituitary alone, suggesting NMDA and DAA may work in synergy to stimulate the release of various hormones.

Anabolic hormones

Anabolic hormones that can contribute to hypertrophy or strength gains include testosterone, IGF and human growth hormone. The response of IGF-1 to resistance training have been observed in the literature to increase (Borst, Vincent, Lowenthal, & Braith, 2002; Marx et al., 2001), or not change at all (Kraemer et al., 1999; Petrella, Kim, Cross, Kosek, & Bamman, 2006; Walker, Kambadur, Sharma, & Smith, 2004). Basal levels of growth hormone tend not to change from resistance training (Craig et al., 1989; Kraemer et al., 1999; Kraemer et al., 1998; Marx et al., 2001). This may be attributed to the large variety in isoforms of GH produced in the body and the limited availability to detect them with current technology. Of all the hormones in the body, testosterone is considered to be one of the most powerful androgenic-anabolic hormones secreted by the body.

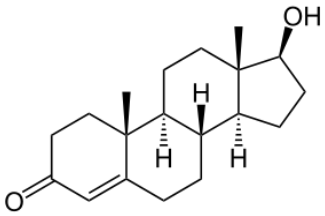


Figure 3 Testosterone

Testosterone ((17β)-17-Hydroxyandrost-4-en-3-one) is a 0.288 kD 19 carbon steroid hormone of the androgen class. It is primarily produced in the Leydig cells of the testes or in smaller quantities in the ovaries and the adrenal cortex. Testosterone is of interest to the strength and conditioning community for direct action on androgen receptors and up-regulation of insulin-like growth factor 1 (IGF-1) (Wu et al., 2007). Interaction of testosterone with the AR causes the AR to activate and bind to the testosterone molecule. Subsequently the AR-testosterone complex cause an upregulation of the rate of transcription, by joining together with selective genes (Kadi, 2008). Besides genomic pathways via AR interaction, research has also identified mechanisms of signal transduction. Through a series of rat experiments using inhibitors of phosphatidylinositol 3-kinase / protein kinase B (PI3K/Akt) pathway and AR inhibition (Basualto-Alarcón, Jorquera, Altamirano, Jaimovich, & Estrada, 2013) it was shown that testosterone initiates Akt phosphorylation via PI3K, which in turn activates the mechanistic target of rapamycin / ribosomal protein S6 kinase 1 (mTOR/S6K1) axis (Basualto-Alarcón et al., 2013). Both signalling pathways (AR and Akt/mTOR/S6K1) play a role together in skeletal muscle hypertrophy. Additionally, the results of this study suggest the likelihood of crosstalk occurring between the two pathways, which would assure an organised response pattern (Basualto-Alarcón et al., 2013).

Testosterone secretion is controlled by a negative feedback loop, as shown in Figure 4. In the healthy young adult, gonadotropin releasing hormone stimulates the release of luteinizing hormone, which stimulates testosterone secretion. As more testosterone becomes available, it will feed back through the negative feedback loop, inhibiting

production to maintain normal testosterone levels. Evidence from small mammal studies suggests exogenous supplementation of DAA accumulates within various tissues along the HPG axis, causing upregulation of testosterone production through various mechanisms.

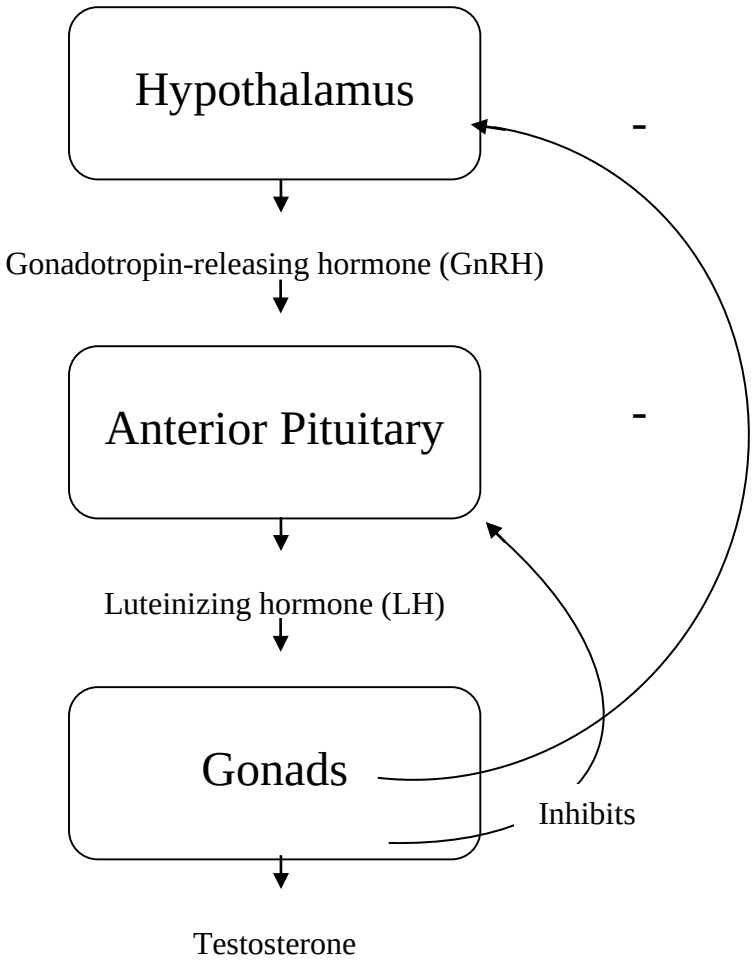


Figure 4 Testosterone Negative Feedback Loop

When DAA accumulates in the testes, the increase in production of testosterone is believed to occur via hCG-induced synthesis. This increase in output occurs from upregulation of the transportation of cholesterol from outside the mitochondrial membrane to the inner membrane (Furuchi & Homma, 2005). The overall rate of testosterone production is limited by cholesterol availability and DAA has been shown to increase the mRNA and protein levels of steroidogenic acute regulatory protein

(StAR), which is a key regulator of the transport of cholesterol from the outer to inner membrane (Nagata, Homma, Matsumoto, et al., 1999). This mechanism first demonstrated in rats has also been identified in humans (Miller & Bose, 2011). Since accumulation of DAA occurs within the rat hypothalamus and anterior pituitary gland causing increased production of luteinizing hormone (A. D'Aniello et al., 1996). Theoretically, testosterone would be indirectly increased (Figure 4). These mechanisms suggest that it is reasonable to believe that testosterone levels could be manipulated, via the supplementation of DAA. The chronic manipulation of basal testosterone could provide a plausible mechanism of hypertrophic and strength improvement.

Mechanisms of hypertrophy

Skeletal hypertrophy is largely a result of the accretion of the myofibril proteins, actin and myosin. The addition of new muscle sarcomeres in parallel increases the cross-sectional size of the myofibril and thus the muscle fibres (Goldspink, 1985). Exercise-induced muscle hypertrophy arises from the accrual of multiple increases in the rate of myofibrillar synthesis rate that occur after each exercise bout across a training protocol (Brook et al., 2015; Damas et al., 2016). Multiple mechanisms have been discovered that help to explain muscular hypertrophy. These are broadly categorised into intrinsic factors within the muscle (e.g. muscle protein synthesis) and systemic factors (e.g. basal hormonal levels).

Intrinsic factors

Hypertrophic mechanisms pertaining to within the muscle are known as intrinsic factors. Intrinsic factors include activation of various signalling pathways (Akt/mTOR,

PA/mTOR, mechanoreceptor), phosphorylation of intramuscular signalling proteins, regulation of messenger RNA (translation initiation), increases in AR content, satellite cell activity and expression of muscle-specific microRNA.

mTOR

One of the most important pathways in the regulation of muscle protein synthesis is the mammalian/mechanistic target of rapamycin complex 1 (mTOR). mTOR and downstream proteins are important regulators of the initiation of mRNA translation (Burnett, Barrow, Cohen, Snyder, & Sabatini, 1998). The process of translation initiation involves the binding of a ribosome to the mRNA (Sonenberg & Hinnebusch, 2009). The stages of translation can be separated into initiation, elongation, termination and ribosome recycling (Sonenberg & Hinnebusch, 2009). Protein synthesis data in rats portrays increased polypeptide initiation, but not elongation suggesting that the initiation of mRNA translation is the rate-limiting step in protein synthesis (Monier & Le Marchand-Brustel, 1982). mTOR is initiated by the phosphorylation of upstream targets including insulin receptor substrate 1 (IRS1), Akt, tuberous sclerosis complex 2 (TSC2). Downstream targets of mTOR include ribosomal S6 kinase 1 (p70S6k), 4E-BP and ribosomal protein S6 (RPS6) (Sonenberg & Hinnebusch, 2009). 4E-BP regulates translation by binding with the eIF4E (cap-binding factor). Activation of mTOR disassociates 4E-BP from eIF4E via phosphorylation, along with the phosphorylation of other cap-binding factors – eIF4G and eIF4A. The freedom of these cap-binding factors allows for them to bind with the capped 5' end of mRNA enabling them to form the translation initiation complex eIF4F. mTOR also phosphorylates p70S6k, which in turn phosphorylates RPS6, which is believed to help with translation initiation (A. M. Gonzalez, Hoffman, Stout, Fukuda, & Willoughby, 2016).

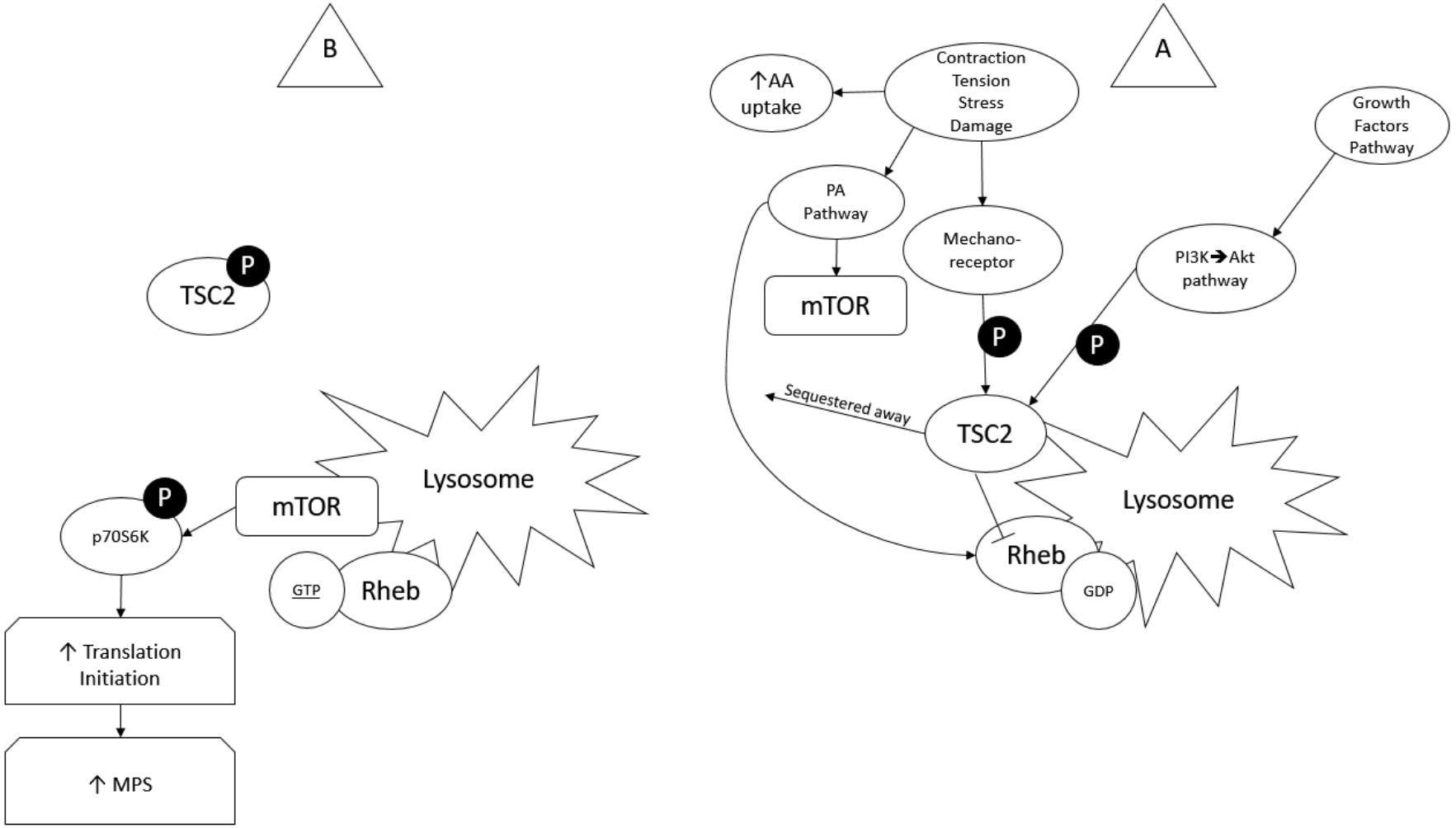


Figure 5 Signalling pathways involved in muscle protein synthesis.

It has been proposed that muscle lysosomes contain an abundance of Rheb and TSC2 at rest. However, the presence of TSC2 keeps Rheb in its inactive, GDP-bound state (Sato, Nakashima, Guo, & Tamanoi, 2009), which also causes mTOR to be inactive (Jacobs et al., 2013). Thus, TSC2 is considered an upstream inhibitor of mTOR. The phosphorylation of TSC2 (Figure 5, A ➔ B) causes it to disassociate from the lysosome and allows Rheb to change to a GTP-bound state. Amino acid availability is thought to help to activate mTOR (Mackenzie, Hamilton, Murray, Taylor, & Baar, 2009). The localisation of mTOR to the lysosome allows for the interaction with GTP-bound Rheb, causes mTOR to activate (Jacobs et al., 2013). Activation of mTOR phosphorylates p70S6k, which upregulates the initiation of translation, increasing MPS rates.

Traditionally mTOR has been explored with respect to activation by the growth factors, insulin and IGF-1. These growth factors bind to their respective receptors causing insulin receptor substrate 1 to recruit PIK3 (Shepherd, Withers, & Siddle, 1998) to phosphorylate PIP2 into PIP3 (Alessi & Cohen, 1998). Phosphorylation of PIP3 then results in the phosphorylation of Akt and Akt phosphorylates TSC2 (Alessi et al., 1997), causing TSC2 to relocate and eventuate in the aforementioned activation of mTOR and subsequent increase in MPS. However, studies have shown that resistance exercise causes either a decrease (Deldicque et al., 2008) or no change in Akt phosphorylation (Adam M Gonzalez et al., 2015), despite phosphorylation of p70S6K, suggesting resistance exercise can activate mTOR independently of the growth factor pathway. Data indicates that post strength training T2C2 is phosphorylated by an unknown kinase, which is theorised to be detected by a mechanoreceptor when the muscle is under load (Figure 5A). Additionally, another recently discovered pathway is related to the production of phosphatidic acid, which

was upregulated in the stretched muscle (Hornberger et al., 2006) and during eccentric exercise (O'Neil, Duffy, Frey, & Hornberger, 2009). PA has been shown to activate mTOR directly by binding to the FRB domain of mTOR (Chen & Fang, 2002). Additionally, an upstream enzyme responsible for the production of PA (PLD1) was suggested to be an effector of Rheb (Sun et al., 2008) (Figure 5A).

Significant downstream targets of mTOR include the kinase p70S6k and the ribosomal protein S6. Rats subjected to electrical stimulation demonstrated a high correlation between the percent increase in muscle mass and the percent change in p70S6k phosphorylation (r=0.998) (Barr & Esser, 1999). Since then this has also been demonstrated in untrained males, whereby increase in Thr389 phosphorylation p70 was significantly correlated with percentage change in 1RM squat strength (r=0.84), whole body FFM (r=0.89), FFM of the leg (r=0.81) and type IIA fibre CSA (r=0.82) (Terzis et al., 2008). Furthermore, this appears to be volume dependent, with a 3x6RM protocol elevating phosphorylation of p70S6k three-fold and a 5x6RM protocol elevating it six-fold (Terzis et al., 2010). The combination of this data suggests that these downstream effectors are important indicative markers of MPS.

Muscle protein synthesis has long been believed to be a driver of muscle hypertrophy. However, there is a lack of correlation between the initial acute rise in MPS and chronic muscle hypertrophy (Mitchell et al., 2014). Recently a close connection between MPS and resistance training-induced muscle hypertrophy has been demonstrated in healthy males (Damas et al., 2016). After ten weeks of resistance training, the integrated muscle protein fractional synthetic rate (0–48 hour, obtained at the 10-week time point) was observed to be highly correlated (r = 0.91) with fibre CSA (Damas et al., 2016) and with vastus lateralis CSA (r = 0.94) (Damas et al., 2016). The greatest absolute response of MPS in this study occurred in the 48 hours after the initial

resistance training bout and also corresponded with the largest observation of muscle damage (as indicated by Z-line streaming) (Damas et al., 2016). Both the response of MPS and the direct marker of muscle damage, after this initial training bout, were not significantly correlated with muscle hypertrophy. Normalisation of MPS to the percentage area containing Z-band streaming (direct damage), resulted in similar observations between the acute response to the initial training bout, the three-week bout and the ten-week bout of resistance training. The data from this study suggests that a novel training stimulus increases absolute MPS to a great extent. However, this increase may be more indicative of repair to damaged proteins, rather than the accrual of additional myofibrils in parallel, which explains the lack of correlation with markers of hypertrophy. By three weeks of resistance training, there are significant correlations between hypertrophy and MPS, and these are augmented further after ten weeks of training (Damas et al., 2016).

Satellite Cells

Skeletal muscle stem cells, more commonly known as satellite cells because they were revealed to be located above the myofiber, beneath the basal lamina. First discovered over 50 years ago within frogs (Mauro, 1961), only in the last decade or two has research begun to better understand the function and role of SC within skeletal muscle. The myocyte is a cell or muscle cell is a multinucleated cell containing many hundreds of nuclei (Dumont, Bentzinger, Sincennes, & Rudnicki, 2015). Each nucleus regulates gene production for a given volume of cytoplasm, within the domain of the nucleus (Allen, Roy, & Reggie Edgerton, 1999). The myonuclear domain hypothesis is a prevailing theory, which describes that the ratio of nuclei to cytoplasm remains relatively constant. As previously outlined the acute exercise bout increases signalling mechanisms that increase protein synthesis, via an enhancement of translational

efficacy (Sonenberg & Hinnebusch, 2009). The net accretion of protein increases the myofiber volume. Satellite cells become relevant in the later phase of myofiber growth, once the myofiber volume increases to a point whereby the current nuclei population cannot support the volume of cytoplasm. At this point, new nuclei are believed to be integrated to support continual growth (O'Connor, Pavlath, McCarthy, & Esser, 2007). This is achieved by proliferation and differentiation of satellite cells (Collins et al., 2005). Through injury (Zammit et al., 2004), growth stimuli (Zammit et al., 2004) and permissive testosterone levels (Kvorning et al., 2015), the cells are activated from their quiescent state, express various transcription factors, Pax7 and MyoD (Zammit et al., 2004), to re-enter the cell cycle, causing the cells to multiply (Siegel, Kuhlmann, & Cornelison, 2011). A unique property of satellite cells is the ability to migrate to the damaged area, a process known as chemotaxis (Bischoff, 1997). Some cells will differentiate into myoblasts and fuse with the damaged myofiber to become available as nuclear donors, thus balancing the myonuclear domain ratio. Others regain a quiescent phenotype to support the satellite cell pool (Zammit et al., 2004).

In mice where SC can be reduced, the abolishment SC has demonstrated conflicting results. One paper demonstrated the same increases in muscle mass and fibre cross-sectional area between normal mice and gene-altered mice (SC 90% depleted) (McCarthy et al., 2011). Contrastingly the data from a more recent study that used an almost identical study design demonstrates that satellite cell ablation prevents overload hypertrophy (Egner, Bruusgaard, & Gundersen, 2016). Egner and colleagues' highlights that the discrepancies between the papers were likely due to the methodology for the determination of hypertrophy. Specifically, the reliance of relative muscle mass as a proxy for fibre hypertrophy and inclusion of regenerating fibres that was used in the 2011 paper, may have resulted in the lack of significant

differences between the groups. SC undeniably play a role in skeletal muscle hypertrophy, despite the debate of if they are essential or not. In humans, studies have shown that change in the myonuclear number correlate with increases in myofiber size (Bellamy et al., 2014; Leenders et al., 2013; Petrella et al., 2006). Conversely, others have demonstrated increases in myofiber size independent of myonuclear addition (Kadi et al., 2004; Mackey et al., 2007; Petrella et al., 2006). This has led to the theory of a myonuclear domain ceiling, which suggests that an absolute or relative threshold for myonuclear addition exists before there is a requirement for subsequent myonuclear addition (Petrella et al., 2006; Petrella, Kim, Mayhew, Cross, & Bamman, 2008). In humans, research also demonstrates that normal testosterone levels are required for the differentiation of satellite cells (Kvorning et al., 2015). Testosterone also has a direct effect on other intrinsic mechanisms.

Androgen Receptor

The anabolic action of testosterone is facilitated by the androgen receptor. Both concentric and eccentric exercise increases concentrations of mRNA for androgen receptors, suggesting increased turnover from resistance training (Bamman et al., 2001). Data indicates that AR fold change is correlated with mean fibre change, despite no significant change in AR protein content pre to post resistance training (Mitchell et al., 2013). Similar results have been observed in young and old men (Ahtiainen et al., 2011). Related to these intrinsic factors are the various conditions and stressors that resistance exercise exposes the muscle to.

Exercise muscle stress

Resistance training exposes the muscle to mechanical tension, metabolic stress, damage to the muscle fibres and surrounding structures. It is not yet clear as to the specific mechanosensor that detects muscle tension and activates intrinsic factors to up-regulate MPS. Plausible locations of the mechanosensor include the myosin filaments, extracellular matrix, the myotendinous junction and the costamere (West, Burd, Staples, & Phillips, 2010). This mechanism is likely linked with muscle damage as it is believed that deformation of proteins within one or more of these structures directly activates mTOR via release of a phospholipid called phosphatidic acid (PA), (You, Frey, & Hornberger, 2012) or via an mTOR-independent pathway involving focal adhesion kinase (FAK) (Klossner, Durieux, Freyssenet, & Flueck, 2009). Muscle damage is believed to be an important driver of muscle hypertrophy. However, the extent of damage that is experienced in some protocols may be unnecessary as the dose response of muscular damage has yet to be determined. Surrounding the muscle cell is the membrane called the sarcolemma. Performing resistance training can cause deformation of the sarcolemma (West, Burd, Staples, et al., 2010), which is also known to release a phospholipid called phosphatidic acid (PA) (O'Neil et al., 2009), which as previously discussed can upregulate mTOR. Furthermore, damage to the myofibres will cause activation of satellite cells to up-regulate hypertrophy via these mechanisms (Zammit et al., 2004).

Metabolite accumulation during resistance training may be a potential up-regulator of downstream growth mechanisms. Isometric training has demonstrated superior hypertrophy in long continuous contractions in comparison to short, intermittent contractions (Schott, McCully, & Rutherford, 1995). The group with longer

contractions also experienced a greater drop in pH, phosphocreatine (PCr) and a greater rise in inorganic phosphate (P_i) and the P_i : PCr ratio. Time under tension volume was matched, suggesting that the group that experienced the greater metabolic stress also experienced greater hypertrophy (Schott et al., 1995). Further evidence of the role of metabolites is seen in blood flow restriction studies. Typically, low load training (<60% 1RM) appears to attenuate the hypertrophic response (Schoenfeld, Wilson, Lowery, & Krieger, 2016), however, the introduction of metabolic stress via blood flow restriction has been demonstrated to create a hypertrophic response in untrained or recreational subjects (Loenneke, Wilson, Marín, Zourdos, & Bemben, 2012) in comparison to low load training. Despite this data, research is not yet clear as to the specific mechanisms that metabolic stress affects to upregulate MPS. Theoretical mechanisms include increased fibre recruitment, elevated hormonal release, altered myokine production, production of reactive oxygen species and cellular swelling (Schoenfeld, 2013). It is plausible that metabolic stress could also play a role in the upregulation of mTOR. In addition to these intrinsic factors, systemic factors also perform a role in the hypertrophic effect.

Systemic factors

Hypertrophic mechanisms pertaining to factors arising from outside the muscle are known as systemic factors. These include testosterone, growth hormone, insulin-like growth factor 1 and interleukin-6 (IL-6) (Mitchell et al., 2013). IL-6 has been suggested as an important marker for future research as it is implicated as a regulator of satellite cell function (McKay et al., 2009). Furthermore, it has been shown to be positively correlated with mean fibre CSA after 16 weeks of resistance training (Mitchell et al., 2013). Other systemic factors, especially hormonal influence, was a

major focus of early research due to the belief that they could predict the hypertrophic potential of a training protocol. The relevance of systemic factors is still debated. However when measured as transient elevations detected in serum the evidence is strongly suggesting they play, more of a permissive role in hypertrophy than predictive of hypertrophic potential.

Early research observed a variety of different acute testosterone responses to various changes in training program variables such as intensity; volume; exercise type; exercise order; rest period; and total time exercised (Craig et al., 1989; Cumming, Wall, Galbraith, & Belcastro, 1987; Gotshalk et al., 1997; Kraemer et al., 1992; Kraemer et al., 1990; Ostrowski, Wilson, Weatherby, Murphy, & Lyttle, 1997). This led early researchers to believe that the acute elevation in anabolic hormones was an important predictive marker of the potential for a resistance training protocol to provide maximum strength or hypertrophy gains. There has been some research that suggests that acute elevation of hormones might play a role in hypertrophic or strength outcomes. In 2001 Hansen and colleagues explored the effects of an arms only training group compared with a legs and arms group. What the authors observed was that the legs and arms group experienced a greater hormonal response in testosterone and growth hormone. This group also observed a higher percentage increase in isometric strength, with no significant differences in dynamic strength observed between the groups. However, as there was a significant difference between isometric strength at baseline (legs and arms were lower), which could have skewed the results as they were reported as a percentage change (Hansen, Kvorning, Kjær, & Sjøgaard, 2001) these results need to interpreted with caution. Transient increases in testosterone from an acute bout of resistance training has been shown to blunt androgen receptor catabolism. Specifically, the experimental group performed a high volume upper body

session before a lower body session, with the control group asked to rest before the lower body session. At three hours post resistance training, androgen receptor responses trended down in the control group. Furthermore, the experimental group was significantly greater in comparison to the control group at this time point (Spiering et al., 2009). Another paper that experimented with this design performed legs before arms in a within-subject study design (Rønnestad, Nygaard, & Raastad, 2011). They found that the experimental group experienced greater increases in hypertrophy of the arms (along with a greater acute response in testosterone and growth hormone) in comparison to the control group. As the participants of this study were untrained, it is possible that early adaptation to resistance training might be affected by temporary increases, as there is no prior exposure to these elevations in hormones. Research also suggests that acute elevations in hormones are not necessary to elicit hypertrophy.

Wilkinson and colleagues demonstrated that in untrained individuals, eight weeks of unilateral resistance training increased whole leg CSA and muscle fibre CSA increases of type IIx and IIa. Despite these positive increases, no changes were observed in any acute hormones measured - testosterone, free testosterone, luteinizing hormone, sex hormone binding globulin, growth hormone, cortisol and IGF-1 (Wilkinson, Tarnopolsky, Grant, Correia, & Phillips, 2006). Research exploring the effect of transient elevations in hormones has failed to show a significant difference in MPS or the phosphorylation of p70S6K. West and colleagues showed similar increases in these markers in both a high hormonal condition and a low hormonal condition (West et al., 2009). The authors followed this research with a training study; the demonstrated that the high hormonal condition was able to increase growth hormone, testosterone, and IGF-1, however, muscle CSA was not significantly different between groups post training. They further reported that muscle fibre CSA (type I and II) also increased to

a similar extent (West, Burd, Tang, et al., 2010). Similar null relationships have been observed when research explores the relationship between the area under the curve and various measures of strength and hypertrophy (West & Phillips, 2012) (aside from a weak correlation with growth hormone and type I fibre CSA). In resistance trained men the acute post-exercise rise in anabolic hormones showed no significant correlations with strength or hypertrophy (Morton et al., 2016).

The growing body of evidence against the acute hormone hypothesis suggests that muscular hypertrophy is largely an intrinsic process. The combination of these studies suggests that normal levels of systemic hormones might play more of a permissive role in the overall training effects of a training stimulus and that transient increases in serum hormone concentrations do not appear to increase this effect. As resistance exercise-mediated elevations in testosterone only last for approximately 30 minutes (Smilios, Pilianidis, Karamouzis, & Tokmakidis, 2003), it is believed these differences in short-term elevations do not provide enough of a stimulus to improve hypertrophy over a training period. Increases in resting testosterone levels may, on the other hand, provide a more feasible mechanism for training-related gains. By elevating testosterone levels chronically this provides a greater chance of AR interaction and testosterone signalling downstream mechanism, which over the period that testosterone is increased, allows increased periods of positive protein balance, and hypothetically result in a larger hypertrophic effect.

Testosterone and hypertrophy

It is evident throughout the human male's life that testosterone plays a major role in regulating muscle mass and growth. Puberty, a time of growth, is associated with

increased levels of anabolic hormones (e.g. testosterone) (Richmond & Rogol, 2007). In healthy young males, levels range between 4.5-10.0 ng/ml (Borst & Mulligan, 2007). The process of ageing causes testosterone levels to decline slowly. Epidemiologic studies demonstrate that testosterone levels rise from the 20th decade where they peak and then trend down each decade at a slow constant rate. This pattern has been demonstrated in data from the Baltimore Longitudinal Study of Ageing (Harman, Metter, Tobin, Pearson, & Blackman, 2001), in community-dwelling men (Ferrini & Barrett-Connor, 1998) and ageing and obese men (Vermeulen, Kaufman, & Giagulli, 1996). It is believed that the reason for the decline in testosterone could be due to a decrease in Leydig cell numbers, cell secretion and increased sensitivity to the negative feedback loop (Borst & Mulligan, 2007). Declines in total testosterone have been associated with an age-dependent increase in body fat percentage, and declines in bioavailable and free testosterone are associated with fat-free mass and handgrip strength (Chin et al., 2012).

Testosterone supplementation is associated with an increase in satellite cell number. This has been demonstrated in young (Sinha-Hikim et al., 2003) and old (Sinha-Hikim et al., 2006) people. Satellite cells (SC) as previously discussed are an important mechanism of continual hypertrophy by regulating the myonuclear domain (Allen et al., 1999). The SC proliferate and move to the damaged region, where they fuse to the damaged myofiber and are eventually integrated resulting in increased myofiber size (Hawke & Garry, 2001). Besides a permissive role in satellite cell activity, a direct mechanism of testosterone-imitated hypertrophy is the binding with the AR on a muscle cell. In the resting state, the AR exists within the cytoplasmic as a complex with heat shock proteins. When testosterone binds with the AR this complex transforms, which allows nuclear translocation of the AR along with AR homodimer

formation (Prescott & Coetzee, 2006). As the AR is now activated within the nucleus, it can bind to androgen response elements that affect target genes. Once bound to these elements the AR dimer can interact with the transcription pre-initiation complex to up-regulate transcription rates which result in an increase in muscle protein synthesis (Heemers & Tindall, 2007). The importance of the AR-testosterone complex is supported by research where ARs were inhibited in rats, occurring in parallel with reductions in muscle hypertrophy (Inoue et al., 1994).

The interaction of testosterone with ARs is not limited to muscle cells, as ARs can also be found on motor neurons. Androgen receptors found within the sciatic nerve of the rat, suggest that testosterone could have potential neurological effects on the peripheral nervous system (Jordan et al., 2002). Data on gerbil studies indicates that testosterone interacting with these AR may maintain the relative size of the motor neurons (Herbst & Bhasin, 2004) throughout normal life. One particular study showed that long-term castration reduced motoneuron size in the gerbils, further experiments demonstrated that testosterone propionate treatment prevented a reduction in motor neuron size (Fraley & Ulibarri, 2002). The presence of androgen receptors has also been determined in human fetal brain tissue, with evidence of the ability of testosterone to provide neuroprotection against serum deprivation (Hammond et al., 2001). With the addition of an aromatase inhibitor, it was also determined that the neuroprotective effects was likely caused by testosterone, rather than indirectly via the aromatization of testosterone to estradiol (Hammond et al., 2001). It is plausible that a secondary benefit of testosterone could also be to provide neural adaptation mechanisms, potentially improving transmission efficiency, which could translate into strength gains.

Neural adaptation and strength

Although resistance training has obvious benefits on fibre hypertrophy, early literature suggested that not all strength observation could be as a result of muscular adaptation. The first indication of neural adaptation was in research where early strength gains were observed before any hypertrophy was identified (Akima et al., 1999; Moritani & DeVries, 1979), suggesting that changes in the nervous system played a role in strength improvements. The brain and spinal cord are known to be highly adaptable, or highly plastic in response to various tasks, such as skill, endurance or strength training. Anatomical changes within the ventral horn of the spinal cord have been observed in rats exposed to resistance training, with an increase in the number of excitatory synapses in spinal motoneurons observed (Adkins, Boychuk, Remple, & Kleim, 2006). Additional results demonstrated that training of a forearm grasping task led to changes in movement representations within the motor cortex. Two groups, skill reaching and load based strength reaching were tested against non-grasping controls. Similar changes were observed in the motor cortex, regardless of the load in the skill and strength reaching groups (Remple, Bruneau, VandenBerg, Goertzen, & Kleim, 2001). Since strength training does not favourably change representation patterns within the motor cortex, then other neural mechanisms must contribute to the observed strength change. Neural adaptation occurs within the body as a result of a training stimulus and is a key explanatory driver of strength development. Multiple mechanisms can adapt within the central and peripheral nervous systems to improve strength. These can be measured via electrical stimulation, which can investigate neural adaptation along various sections of the pathway, from the brain to muscle. The process of voluntary movement involves an electrical signal travelling from the motor cortex down the corticospinal tract, connecting with the spinal cord, where the upper motor neuron

synapses with interneurons and alpha motor neurons (α-MN) within the ventral horn at the relevant location of the spine. The action potential (AP) then travels down the α-MN axon which innervates muscle fibres via the motor end-plate and causes them to contract (Siegel, Sapru, & Siegel, 2014). Located within the muscle are muscle spindles which monitor the length of a muscle and how it changes. The sensory information of the muscle spindles is relayed via the type Ia afferent fibres to the α-MN, mediated by an interneuron, which controls presynaptic inhibition (Zehr, 2002). Improvements in excitability of the Ia afferent pathway, from increased efficiency or reduction of presynaptic inhibition, are believed to increase the firing rate of the α-MN resulting in greater force production. This reflex pathway is researched in the tibial nerve and calf muscles and is explored by changes in the Hoffman reflex.

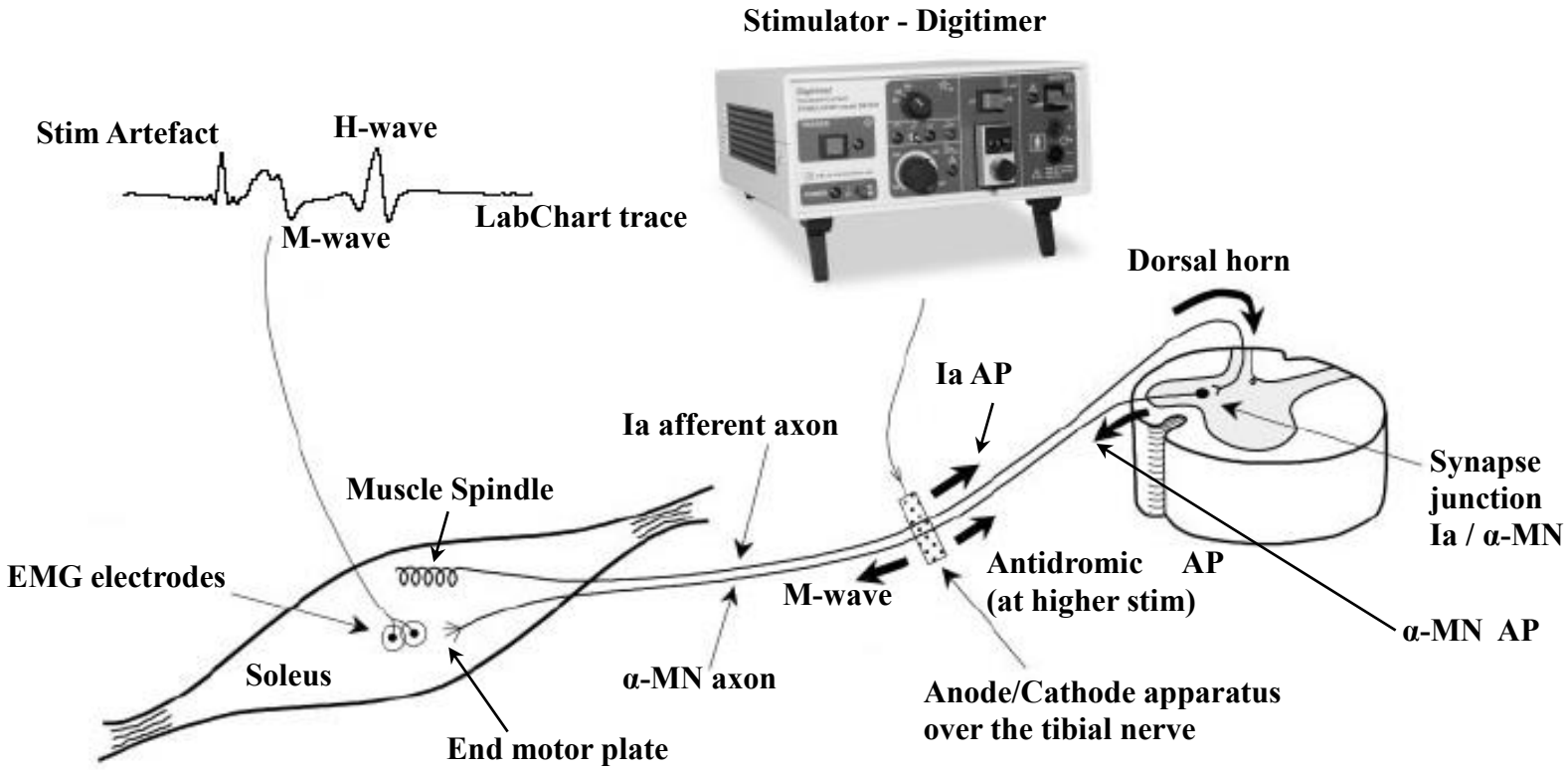


Figure 6 Overview of the neurophysiology involved with the Hoffman reflex, adapted from Aagaard et al. (2002b).

The Hoffman reflex (H-reflex) is electrically evoked by stimulating a peripheral nerve and observing changes in the recorded response (EMG) in the muscle innervated by that nerve. Despite direct connection of Ia afferents to the α-MN, it is important to highlight that changes in the H-reflex do not necessarily reflect excitability of the motoneuron pool because of the effect of presynaptic inhibition (Zehr, 2002). Rather the size of the H-reflex reflects the efficiency of the Ia reflex pathway, including potential mechanisms of presynaptic or postsynaptic inhibition. At low-intensity electrical stimulation, where the H-reflex is observed, action potentials are preferentially recruited within the Ia afferents, due to the relatively larger diameter of this sensory axon. These signals travel up the Ia afferent to the spinal cord, via the dorsal root ganglion and elicits excitatory action potentials within the α-MN. The resultant H-wave is recorded approximately 30-40ms later (Aagaard et al., 2002b). As electrical stimulation is increased the thinner fibres of the α-MN are also stimulated causing action potentials to spread in both directions (Figure 6). The signals travelling down the α-MN are recorded as a direct motor response (M-wave). Signals travelling antidromically up the α-MN, collide with the elicited H-wave signals, which eventually cause the H-reflex to dissipate as the M-wave gets progressively larger up to the maximal motor response (M_{max}) (Aagaard et al., 2002b). The change in this pathway, as a result of training, is influenced by the method of how it was elicited and the type of training that was implemented. When H-waves are explored during a complete rest protocol, no changes are observed during hopping training (Voigt, Chelli, & Frigo, 1998), electrical stimulation training (Gondin, Duclay, & Martin, 2006), isometric training (Del Balso & Cafarelli, 2007), eccentric only training (Duclay, Martin, Robbe, & Pousson, 2008), and strength training (Aagaard et al., 2002b; Holtermann et al., 2007; Lagerquist et al., 2006). With a clear lack of change

observed during rest it is believed that changes in H-reflex should be measured during muscle contraction either at MVC or a pre-determined percentage of MVC (tonic contraction). Aagaard and colleagues found increased normalised H-reflex amplitude during a ramped MVC (Aagaard et al., 2002b). Light plantar flexor training (~50% 1RM) increased H-reflexed recruited during a 10% tonic contraction, at 5% of the maximum motor response (M_{max}) (Lagerquist et al., 2006). Three weeks of isometric training resulted in increased H-reflexes at submaximal contractions of 20% and 60% approximately 20% M_{max} (Holtermann et al., 2007). Despite a number of studies showing improvements in this reflex arc, some studies have found no change in H-reflexes: 20% M_{max} at 100% MVC (eccentric focused training) (Nordlund, 2010), and 10% M_{max} at 20% MVC (maximal strength training at 85-90% 1RM) (Fimland, Helgerud, Gruber, Leivseth, & Hoff, 2009). With such a wide variety of testing methods used in the literature, it can be difficult to draw solid conclusions from the available research. One method to help counteract these discrepancies is to map the entire ascending limb of the H-reflex recruitment curve. The H-reflex curve is fitted to a sigmoid function (Klimstra & Zehr, 2008) and a number of useful variables can be determined including the: maximum slope of the curve, current at the threshold of H-reflexes, current at maximum H-reflex and the current at 50% of H-reflex max. This allows excitability of the H-reflex to be assessed at multiple intensities (Vila-Cha et al., 2012). When evaluated in this fashion it was observed that endurance training decreased the current required at H-reflex threshold, 50% H-reflex max and during H-reflex max, suggesting improved excitability of the Ia reflex arc at low and high threshold motor units (Vila-Cha et al., 2012). The strength training group however only experienced a reduction in the current at H-reflex threshold, suggesting that improvements from strength training occurred only at the lower recruited motor units

(Vila-Cha et al., 2012), which is in line with the aforementioned research that observed increases at H-reflex intensities at or lower than 20% M_{max}. These studies as a whole suggest that spinal plasticity of the Ia afferents can occur with various training modes within novices. Spinal plasticity is not the only explanatory mechanism for improvements in strength and power; another important mechanism is increased cortical drive.

The enhancement of neural drive from the motor cortex is an acknowledged mechanism of strength and power improvements. Nerual drive can be investigated by measuring changes in volitional waves (V-Waves). Similar to the Hoffman reflex, V-waves are elicited using a supramaximal electrical stimulation of the relevant nerve during maximal contraction (Aagaard et al., 2002b). The supramaximal stimulus (~130-150% of M_{max}) ensures action potentials are travelling antidromically up the α-MN axons. As this occurs simultaneously with an MVC, neural drive from the motor cortex, elicits efferent motor impulses within the α-MN, which collide with the antidromic action potentials, causing a cancellation effect. Usually, at M_{max} or higher stimulus levels, the H-reflex response is nullified from signals travelling antidromically in the α-MN. However, this cancellation effect caused by the efferent motor impulses occurs before the H-reflex action potentials are elicited in the α-MN axon (Figure 7). This cancellation allows for some of the evoked H-reflex response to be recorded as a V-wave, denoted as such to indicate that it was elicited during voluntary contraction. Consequently, it is believed that an increase in the number of efferent impulses, or frequency of impulses from the motor cortex will result in improved chance of collision between antidromic and efferent action potentials, manifesting as a larger recorded V-wave (Aagaard et al., 2002b). The augmented neural drive is a plausible mechanism for a downstream increase in motor unit

discharge rate. Strength and power improvements have been observed concurrently with increased maximal firing frequency of single motor units in dorsiflexion ballistic training (Van Cutsem et al., 1998). Further research has indicated that motor unit discharge rate increased by ~11% in vastus medialis and vastus lateralis from 6 weeks of resistance training, in comparison to endurance training whereby MU discharge rate decreased by ~11% in these muscles. (Vila-Chã, Falla, & Farina, 2010). Early V-wave research compared the response between resistance trained individuals and novices and found that the experienced weightlifters had 70% higher V-wave response (Sale, Upton, McComas, & MacDougall, 1983). Increases in voluntary torque, in parallel with increases in the V-wave response, have been observed from various training modalities, including heavy periodised resistance training (Aagaard et al., 2002b), neuromuscular electrical stimulation training (Gondin et al., 2006), isometric training (Del Balso & Cafarelli, 2007), eccentric focused training (Duclay et al., 2008; Nordlund, 2010) and transfer from multi-joint strength training to the calf muscles (Fimland et al., 2009). These studies indicate that in novice populations, a significant mechanism of improved strength is increased neural drive to the muscles, especially during early stages of training. Currently, there are no training studies investigating if this mechanism is still apparent in a resistance trained population, or if neural excitability plays a larger role in this population.

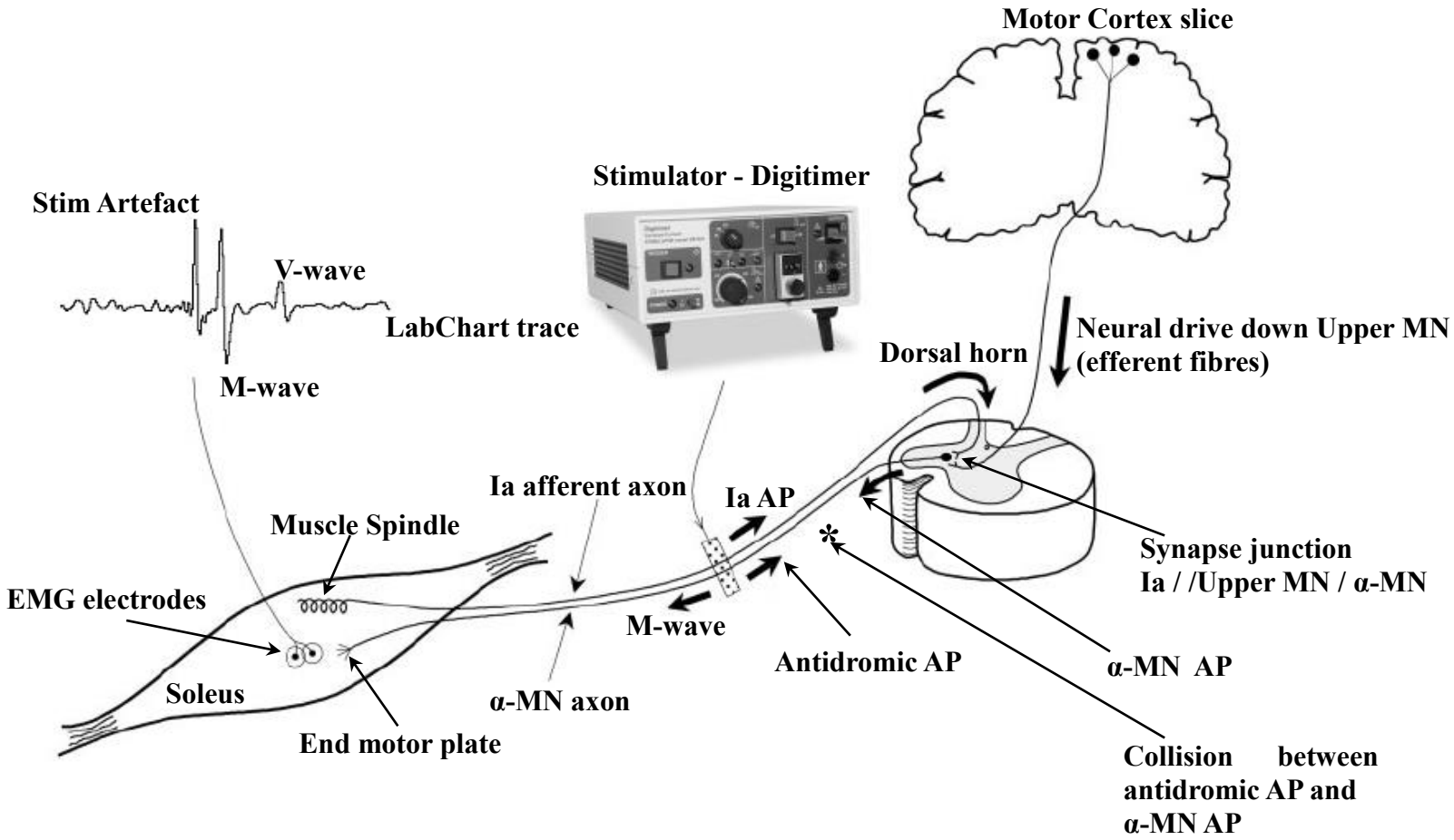


Figure 7 Overview of the neurophysiology involved in eliciting V-waves, adapted from Aagaard et al. (2002b).

Relationship between testosterone and corticospinal pathway

Research has shown that a relationship exists between testosterone manipulation and the adaptation of the corticospinal pathway, in novice trainers (Bonifazi et al., 2004). Healthy males were exposed to an intramuscular injection of hCG, whereby testosterone increased from 4.96 to 8.73 ng/ml 48 hours post injection concurrently with a threefold increase in estradiol. Participant's corticospinal pathway was tested with transcranial magnetic stimulation (TMS). This methodology involves stimulation of the motor cortex with magnetic coils to evoke motor potentials (MEP) within the right FDI muscles. They observed a decrease in the threshold of the cortical response, but no change in the slope of the MEP. A decrease in the threshold represents an increase in the excitability of the lowest threshold motor units (Bonifazi et al., 2004), or an increased output for a given input. They were also able to test soleus H-reflex in two subjects, who experienced no change in the threshold or slope of the ascending limb (Bonifazi et al., 2004). These results suggest that increasing testosterone can increase excitability of the corticospinal tract. The lack of change within the soleus H-reflex parameters suggests that the observed decrease in motor threshold did not occur from changes within the Ia afferent reflex arc, and represented an increase in cortical drive. Since this methodology was only tested in two of the subjects (n=22) and the methods investigated two different motor neuron (MN) pathways (soleus vs. the first dorsal interosseous), spinal adaptation cannot be ruled out as a mechanism for the reduced MEP threshold stimulus. Twenty days' later testosterone and estradiol levels had returned to baseline, along with the observed changes in the MEP threshold. This response between cortical adaptation and hormones provides a plausible mechanism for strength improvements observed in resistance trained populations who tend to have

higher basal testosterone levels. If a larger dosage of d-aspartic acid can increase testosterone levels with this population, then it is reasonable to believe that preferential strength improvements could occur via improved excitability of cortical or supraspinal pathways.

D-Aspartic acid and direct neural mechanisms

Supplementation of DAA may have a direct mechanism of action on neural adaptation which moderates changes in strength. Research supports the notion that DAA has a role as a neurotransmitter or neuromodulator (A. D'Aniello, 2007). Research on chickens and rats (Dunlop, Neidle, & McHale, 1986; Neidle & Dunlop, 1990) indicates during embryogenesis a significant increase in the concentration of DAA within the brain is evident, suggesting a role in central nervous system development. The research to date also suggests that DAA fills many of the criteria for a neurotransmitter, or act as a neuromodulator (Ota, Shi, & Sweedler, 2012; Spinelli et al., 2006). D-aspartic acid has been demonstrated to be present in high concentrations within the synaptic vesicles of axon terminals observed in prepared synaptosomes from the rat brain, and the optic lobe from European squid (S. D'Aniello et al., 2011). Further experiments showed that DAA significantly increased cAMP levels, in rat synaptosomes (S. D'Aniello et al., 2011), as well as *in vivo* increases in cAMP levels, when DAA was injected into live *Aplysia limacina* (Spinelli et al., 2006). Cyclic AMP is a molecule derived from AMP, which utilises the second messenger pathway to allow signalling from one neuron to another. This suggests that DAA signalling, like other neurotransmitters, is mediated by cAMP (S. D'Aniello et al., 2011). An important property of neurotransmitters is the ability to be synthesised within the neuron. The discovery of the existence of the enzyme DAA racemase suggests that neurons can

produce DAA from L-aspartic acid (S. D'Aniello et al., 2011). Additionally, literature has demonstrated that DAA is released from synaptosomes following artificial stimulation with potassium ions (S. D'Aniello et al., 2011). These experiments as a whole support the notion that DAA could play a role as a neurotransmitter. It is not clear from the research if supplementation of DAA in humans could be utilised by the central or peripheral nervous system. However, a plausible mechanism for the uptake of free DAA within the blood is via the L-glutamate transporter system. DAA has previously been observed to have the ability to utilise the L-glutamate transporters (Kanai & Hediger, 1992; Koyama et al., 2005), which provides a means for free DAA to enter via the glia (Spinelli et al., 2006). As the current animal literature focuses on the effects of DAA within brain tissue, it is not clear if the adaptation in humans can even occur, or if it will be exclusively supraspinal in nature, or include adaptation within spinal reflex pathways. The aforementioned research suggests that DAA has the potential to directly affect neural adaptation mechanisms, potentially resulting in power or strength gains.

Resistance training and basal testosterone

Novice populations

The effects that resistance training has on testosterone levels in individuals new to resistance training is equivocal, with some research indicating increases (Izquierdo et al., 2006; Santtila, Kyröläinen, & Häkkinen, 2009; Staron et al., 1994) and other research observing no significant change (Ahtiainen, Pakarinen, Alen, Kraemer, & Häkkinen, 2003; Craig et al., 1989; Hansen et al., 2001; Petrella et al., 2006). The variability of results within the novice literature may be attributed to differences in

training parameters, volume, intensity and frequency of training manipulated in these studies. The relative importance of testosterone during early stages of training is not clear. Potentially in a novice population, other mechanisms such as mechanical deformation of muscle fibres, immune activity, or neural adaptation (Aagaard et al., 2002b; West, Burd, Staples, et al., 2010) may be driving strength and hypertrophy over and above any potential benefits of increased basal testosterone levels. Additionally, normal levels of testosterone would be adequate to initiate downstream signalling events enough to observe some training gains in this population. The training literature in novice individuals ranges from 8 weeks to 6 months, which may not be long enough to initiate an adaptive increase in basal testosterone. This elevation in basal testosterone appears to take at least a year to manifest, as higher resting testosterone levels within trained populations are observed in men with one or more years training experience (Häkkinen, Pakarinen, & Alen, 1987; Häkkinen et al., 1988b; Häkkinen, Pakarinen, et al., 1985; Willoughby & Leutholtz, 2013; Willoughby et al., 2014). Despite this, some research in novice populations has observed increases in testosterone over shorter timeframes (Izquierdo et al., 2006; Santtila et al., 2009; Staron et al., 1994).

In 1994 Staron and colleagues conducted a resistance training protocol in relatively young men and women (Staron et al., 1994). Participants (men and women) performed one week of familiarisation training followed by eight weeks of high-intensity training. Their training resembled a daily undulating periodisation model, incorporating failure training. One session involved an intensity at 10-12 repetitions, the other involved failure at 6-8 repetitions and every fortnight involved a session of one repetition maximum (1RM) testing. Basal testosterone was increased by the fourth week (~5.76 to ~7.49 ng/ml) and stayed elevated throughout the remainder of the eight weeks. A

positive relationship was also observed between the training men's testosterone and the percentage of IIa and IIb muscle fibres, (R^2=0.39 and R^2=0.46 respectively) indicating that higher basal testosterone levels are directly associated with type II muscle fibres in novices. Another study exploring basal testosterone in novices was conducted in Basque ball players over 16 weeks, investigated failure vs. non-failure training (Izquierdo et al., 2006). This study periodised training, decreasing volume and increasing intensity, at each six-week block. The intensity of the first block was 10RM, the second utilised 6RM and the last utilised 5RM with low-intensity ballistic training. The non-failure group observed an increase in testosterone at week 11, which regressed back to baseline by week 16. It is possible that the drop in volume, implemented to prevent overtraining, actually caused the increase in testosterone to return to baseline. Similar gains were observed between groups in 1RM strength (upper and lower body), upper body power, and lower body endurance test. The failure group experienced larger gains in an upper body endurance test, whereas the non-failure group experienced larger gains in lower body power test. Potentially the increase in testosterone observed may explain the increase in squat power, with testosterone levels influencing neural adaptation directly via increased interaction with androgen receptors within neurons at the supraspinal or spinal level (Hammond et al., 2001). Alternatively, the failure based training could have caused both the change in power and the fluctuations in testosterone, with the testosterone change not the true cause of power adaptation.

Research in mixed mode training has also observed increased testosterone as a result of training (Santtila et al., 2009). Conscript soldiers were recruited into an 8-week study involving three experimental groups, basic training (BT) only, BT and resistance exercise, BT and endurance training. Maximal isometric force increased in the strength

and endurance groups and total testosterone increased in all groups. The BT and resistance exercise group experienced a reduction in body mass and fat mass, and a decrease in ultrasound thickness of the triceps brachii thickness, suggesting a slight decrease in hypertrophy. A confounding factor of this study is the basic training that was provided in each group, as the high volume of endurance-based military training, may have caused the reduced hypertrophy gains that were expected in the resistance training group (Santtila et al., 2009). Despite similar increases in total testosterone across all groups, the higher leg volume in the strength and endurance groups appeared to provide the greatest stimulus for increased strength of the soldiers in this study. This again highlights that in individuals new to training the importance of testosterone may be surpassed by the novel training stimulus, which is improving training gains via alternative mechanisms (Aagaard et al., 2002b; A. M. Gonzalez et al., 2016; West, Burd, Staples, et al., 2010). Not all novice research has observed increased testosterone from a training bout.

Early research into basal hormonal changes from training did not find a difference in testosterone levels in both young and old (Craig et al., 1989). Participants trained for 12 weeks, three days per week, using weight machines. No change in testosterone levels was observed in the young group, 8.46±0.58 to 8.27±0.67 ng/ml, yet the participants increased their lean body mass. With these values in the upper clinical normal range – 2.75 – 10 ng/ml (Bhasin et al., 2008) – it is possible that there was not much room for testosterone to increase further, already providing maximum benefits. Another periodised study conducted over ten weeks found in both young and old participants, significant improvements in 1RM squat, increased CSA of the thigh, but no change in total testosterone levels (Kraemer et al., 1999). A study in 2001 primarily explored the effect of the acute hormonal response and also observed lasting changes

in hormones. Researchers used arms-only training, and legs plus arms training (Hansen et al., 2001), with subjects acting as their own controls. Previous research had demonstrated that the hormonal response to a resistance training session was proportional to the size of the muscle volume that was activated, as well as the intensity of the session (Häkkinen & Pakarinen, 1993; Kraemer et al., 1990). Thus Hansen et al. designed this study to expose the arms to two different hormonal environments. The group that utilised arm and leg training observed better percent change improvements in isometric strength in the trained arms, as well as an increase in the acute hormonal response. The authors attributed the acute hormonal response to the significantly greater gains. However, these results have to be interpreted with caution. Baseline values in the combined group were significantly lower compared to the other group, inflating the percentage change results. With respect to the change in resting testosterone, the arms training group (n=8) experienced a trend for an increase, from 6.95 to 8.27 ng/ml ($p>0.05$, $d=0.525$), whereas the legs and arms group (n=6) did not change, 6.37 to 6.28 ($p>0.05$, $d=0.026$) ng/ml. Potentially the study was underpowered to observe a change in resting testosterone. The variability between these groups suggests that, as a beginner, there may be a high individual adaptability to training stimuli, which could be related to genetic endowment. Another study that supports the theory that increases in testosterone levels are less important in novices was conducted over a longer period (21 weeks) (Ahtiainen et al., 2003). The novice group followed a structured and supervised protocol of whole body strength training twice per week. No changes in total testosterone or free testosterone were observed in this group over the entire study period. However, the group did experience increases in the cross-sectional area of the quadriceps and improvements in strength of the right leg. This study highlights that the apparent adaptation in basal testosterone levels, observed in

resistance trained populations, may take time (longer than 21 weeks). Alternatively, since participants were only trained twice a week, it is possible that a greater training stimulus may be required to affect an increase in resting testosterone. Furthermore, it demonstrated that the training stimulus was enough to provide gains in hypertrophy and strength, absent from an increase in testosterone. Multiple studies have also explored the effects of resting testosterone change in the elderly. Research conducted within this population, appears to show no observable change in testosterone levels with resistance training, despite increases in training goals (Craig et al., 1989; Häkkinen et al., 2002; Häkkinen, Pakarinen, Kraemer, Newton, & Alen, 2000; Petrella et al., 2006; Reaburn et al., 1997). This is likely caused by the downregulation of testosterone production that occurs later in life, as well as an increased sensitivity to negative feedback mechanisms of the HPG axis (Figure 4) (Borst & Mulligan, 2007), which may overshadow any potential increases occurring from the training stimulus. Also as the elderly experience loss of muscle mass and strength detriments, as they age, it is likely that the training stimulus is enough to see measurable gains, regardless of the hormonal milieu. These data suggest that the testosterone adaptation to resistance training is variable within novices, and may play less of a role than in resistance trained men.

Table 1 Literature in novices on basal hormones during resistance training. Abbreviations: total testosterone (TT), free testosterone (FT), resistance training (RT), power training (PWR, men (M), women, (W), young (Y), old (O), arms only (A), legs plus arms (LA), non-significant change (NSΔ), ↑/↓ (significant change, increase / decrease), body mass (BM), body fat percentage (BF%), correlation (&), one repetition maximum (1RM), cross-sectional area (CSA), integrated electromyography (iEMG), average (AVG).

Year	1st Author	Name	Population information	Study Length	Training	Hormones	Other relevant results	AVG TT ng/ml ± SD
1989	Craig	Effects of progressive resistance training on growth hormone and testosterone levels in young and elderly subjects	Y O	24w 3d.w	RT	NSΔ TT	↓ BF% ↑ BM	8.46±0.58 8.27±0.67 7.26±0.44 6.86±0.05
1994	Staron	Skeletal muscle adaptations during early phase of heavy-resistance training in men and women	M/W	8w 2-3d.w	RT	M: ↑ TT @w 5,7,9	↑ dynamic STR Type II fibres & TT	w1 5.65±0.45 w5 7.32±1.18 w7 7.83±1.23 w9 7.55±0.84
1997	Reaburn	Serum testosterone response to high-intensity resistance training in male veteran sprint runners	Sprinters	8w 3d.w	RT	NSΔ TT	↑ 1RM, ↑ peak torque	5.82±0.86 (SEM) 5.53±0.81(SEM)
1999	Kraemer	Effects of heavy-resistance & hormonal responses younger vs. older men	Y O	10w	RT Periodised	NSΔ TT	↑ Squat 1RM, ↑ CSA thigh	Y 5.70±0.65 5.78+0.49 O 4.61±0.43 4.57±0.36
2000	Häkkinen	Basal concentrations and acute responses of serum hormones and strength development during heavy resistance training in middle-aged and elderly	40y M/W 70y M/W	6m	RT& PWR	NSΔ TT, FT 0-6m	↑ 1RM, ↑ iEMG	4.87±1.11 4.78±0.81 5.27±1.59 4.83±1.33
2001	Hansen	The effect of short-term strength training on human skeletal muscle - physiologically elevated hormone levels	A LA	9 w 2d.w	RT	NSΔ TT		6.95±1.84 8.27±3.34 6.37±3.83 6.28±3.29

Table 1 (continued) Literature within novices exploring basal hormones during resistance training. Abbreviations: total testosterone (TT), free testosterone (FT), resistance training (RT), power training (PWR) basic training (BT), men (M), women, (W), young (Y), old (O), physically active (PA), bodybuilders (BB), weightlifters (WL), non-significant change (NSΔ), ↑/↓ (significant change, increase/decrease), body mass (BM), body fat percentage (BF%), correlation (&), one repetition maximum (1RM), cross-sectional area (CSA), average (AVG).

Year	1st Author	Name	Population information	Study Length	Training	Hormones	Other relevant results	AVG TT ng/ml
2002	Häkkinen	Effects of heavy resistance/power training on maximal strength, muscle morphology, and hormonal response patterns in 60-75 y old men and women	O 60-75 y	0-12w 13-24w 2d.w	RT PWR	NSΔ TT NSΔ TT	↑ 1RM, ↑ FFM, ↑I, IIa, IIb ↓ BF%	4.21±2.10 3.69±1.24 3.69±1.24 4.21±2.74
2003	Ahtiainen	Muscle hypertrophy, hormonal adaptations and strength development during strength training in strength-trained and untrained men	(PA) (BB, WL)	21w 2d.w	RT RT mix	NR ↑ TT, FT 0-14 ↓ TT, FT 14-21	↑ CSA ↑ MVC TT & MVC (R=0.84)	NR NR
2006	Izquierdo	Differential effects of strength training leading to failure versus not to failure on hormonal responses, strength, and muscle power gains	Basque ball players	16w 2d.w	RT-F FT-NF Periodised	NSΔ TT NSΔ TT 0-16 ↑ TT, 0-11	↑ PWR, 1RM, ↑ PWR, 1RM	NF: w0 8.29±1.40 w6 8.32±1.44 w11 9.30±1.70 w16 8.19±1.64
2006	Petrella	Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women	Y, 20-35y O, 60-75y	16w 3d.w	Leg training	NSΔ TT	↑ myofiber hypertrophy	4.81±0.50 4.42±0.46 4.32±0.28 4.52±0.30
2009	Santtila	Serum hormones in soldiers after basic training	Military conscripts	8w	BT + RT periodised	↑ TT 16.6%	↑ MVC	4.87±1.19 5.50±1.12

Resistance trained populations

In trained populations testosterone levels are higher than in novices (Häkkinen et al., 1988b; Häkkinen, Pakarinen, et al., 1985; Willoughby & Leutholtz, 2013; Willoughby et al., 2014), suggesting that the increase in testosterone could be a slow adaptive response to the continual training stimulus. Pre to post changes in testosterone levels from the resistance trained literature have observed increases (Ahtiainen et al., 2003; Häkkinen et al., 1988b; Häkkinen, Pakarinen, et al., 1985) and other research has demonstrated that testosterone is not influenced (Alen, Pakarinen, Häkkinen, & Komi, 1988; Häkkinen et al., 1987; Häkkinen, Pakarinen, et al., 1985). The lack of change in some of these studies may be attributed to the fact that these men could be at their genetic ceiling for resistance training mediated testosterone adaptation. It is evident however that variations appear to occur within studies which explore multiple data time points (Ahtiainen et al., 2003; Alen et al., 1988; Häkkinen et al., 1987; Häkkinen et al., 1988b; Häkkinen, Pakarinen, et al., 1985). Furthermore, these changes in testosterone appear to coincide with changes in training volume and give rise to positive relationships between testosterone and strength or power changes.

The first long-term training study in resistance trained men was conducted in 1985, by Häkkinen and colleagues, over 24 weeks (Häkkinen, Pakarinen, et al., 1985). This study involved three groups, two training and one control. The RT group performed heavy resistance strength training three times per week in the barbell back squat. Loads and volume were progressively increased monthly, and during the third, fifth and sixth month, heavy eccentric training was included. The power (PWR) group also performed training three days in the week, which included explosive power exercises (e.g. explosive weighted jumping) and strength exercises at moderate intensities (60-80%

1RM) for the arms, legs and trunk. No changes were observed in testosterone levels in the RT group. However, the PWR group noted significant increases by eight weeks (~19%), which stayed elevated till the 16th week, decreased back down by week 20 and returned to baseline by the 24th week. The differences observed in the experimental groups suggests that this style of periodisation, whereby intensity and volume are both increased, may not positively influence testosterone levels. The positive improvement in testosterone observed in PWR could be attributed to the power training element, the relative lighter intensities, or a combination of both. The authors also explored changes in the testosterone/cortisol ratio (T:C), which increased in both groups, over weeks 0-8 and 0-16 and was significantly correlated with maximal isometric force (RT, R=0.86; PWR, R=0.79) during the last four weeks of training. It should be noted that in RT this period marked an overall plateau in isometric force improvement and a large variability in the group data, likely resulting in the direct relationship with T:C. In the PWR group the increase in T:C from weeks 20-24 was preceded by a drop in T:C from weeks 16-20. A possible explanation for these results is that the progressive increase in the volume of jumping and strength training caused a transitory drop in testosterone, which may have recovered as participants got used to the volume. In the men where this recovery was most pronounced, larger improvements in isometric force were observed during this phase. From this paper, it appears that levels of testosterone can fluctuate throughout a training program with varied intensity and volume, and in those individuals whose hormonal profile adapts favourably during a stressful cycle of a training bout, greater improvements in isometric strength may be observed.

There is also evidence in the literature that testosterone levels do not appear to change over long-term training (Häkkinen et al., 1987). When weightlifters were observed in a 12-month follow-up study, their baseline levels (5.13±1.44 ng/ml) did not

significantly change ($p>0.05$, $d=0.756$) by the end of the 12 months (6.37±1.82 ng/ml). The observed effect size suggests that this study could have been underpowered to observe a significant change in testosterone. The authors noted marked decreases in testosterone (~23%) during the preparatory phase of the weightlifter's main competition, which may have also contributed to the lack of overall change observed in testosterone levels. Additionally, luteinizing hormone was observed to be increased from baseline at 4, 8 and 12 months. This approximately occurred around the primary competition, coinciding with the end of the 2-week intensive preparatory phase and likely resulted as a protective mechanism of the HPG axis to prevent testosterone levels decreasing too low to be able to recover from the intensive phase. Importantly this data suggests that increasing volume can negatively impact resting testosterone levels. Serum testosterone/SHBG ratio (T:SH) also decreased during this 2-week intensive phase and was positively correlated ($r=0.63$) with changes in clean and jerk results. This data also suggests that during a stressful phase of a program, individuals whose hormonal profile is less affected by the relative demand of the training might gain greater improvements in strength and power, highlighting the importance of testosterone levels during demanding phases of training. If it is possible to artificially increase testosterone levels, providing a buffer during these stressful periods, the research suggests that this could result in larger training gains.

Progressive increases in total testosterone were observed with continued long duration training (Häkkinen et al., 1988b). This is the longest training study in resistance trained men (~7 years trained), conducted over a two-year period. In this study, weightlifters performed their regular individualised training programs developed by their coaches, while researchers periodically observed changes in hormones, strength and neuromuscular changes. They observed significant increases in testosterone levels from pre to post, as well as significant increases in the yearly means. Testosterone-to-SHBG ratio increased between the annual means, with a positive correlation between this ratio from the second year and the percentage change in averaged concentric power index (r=0.84) also detected. These stronger relationships with hormones and strength, or hormones and power, appears to be more common in resistance trained populations. As training age increases, the importance of the hormonal milieu may increase, in comparison to the importance of intrinsic factors involved in muscular strength and power (A. M. Gonzalez et al., 2016). Despite no detected changes in T:C pre to post or between annual means, there was a significant correlation between percentage change in T:C and percentage change in the average level of maximal force. The authors theorised that this ratio is an indication of the balance of an individual's anabolic and catabolic status, and may be an important indication of an elite athlete's potential for strength improvement (Häkkinen et al., 1988b).

Another study in the literature explored changes in basal testosterone levels in strength athletes (Ahtiainen et al., 2003). This study observed two groups over a 21-week strength training period. These groups were strength athletes and non-strength athletes (novices). The novice group performed a structured controlled protocol, whereas the strength athletes, who were a mixture of bodybuilders and weightlifters, conducted their own individualised RT programs. Despite a high variability in the programs, the

RT group performed strength training three days per week, training different divisions of the body each day at a repetition range between 6-12 reps. It was found that from baseline testing, testosterone increased by week 7, and again at week 14 before it returned to the near baseline levels by week 21. This corresponded with an increase in total volume (calculated from training diaries), during the middle seven weeks and a slight decrease in the last seven weeks (Ahtiainen et al., 2003). This is in line with previous research discussed, whereby alterations in testosterone appear to mirror training volume changes (Häkkinen et al., 1987; Häkkinen et al., 1988b). This could be a natural adaptation within resistance trained men, to assure testosterone-mediated hypertrophy and recovery is maximised during high stress or high volume training bouts. The authors also found a positive relationship with the testosterone levels (overall training period average) and percentage change in isometric force ($p<0.01$, $R=0.84$), despite no significant change in isometric force (Ahtiainen et al., 2003). Some participants experienced a decrease in isometric strength, while only two increased by greater than 10 %. Potentially this demonstrates overtraining in some of the participants. It also suggests that the individuals who were able to maintain a higher level of testosterone throughout the study period were better able to preserve or increase isometric strength. The aforementioned literature suggests that our current understanding of the ability of resistance training to affect resting hormonal levels in trained men is limited. Furthermore, there are no studies that specifically explore the time course of the change in basal hormones that occurs from prolonged training (novice to advanced). More research is required in resistance trained men, to fully elucidate the effects of testosterone on training gains pertaining to strength, power and hypertrophy.

Table 2 Literature in trained populations on basal hormones during training. Abbreviations: total testosterone (TT), free testosterone (FT), luteinizing hormone (LH), sex-hormone binding globulin (SHBG), cortisol (CORT), training experience (TE), resistance training (RT), power training (PWR, Olympic weightlifting (OLY), non-significant change (NSΔ), ↑/↓ (significant change, increase / decrease), body mass (BM), body fat percentage (BF%) maximal isometric force (MIF), (SJH), correlation (&), not reported (NR), average (AVG).

Year	1st Author	Name	Population information	Study Length	Training	Hormones	Other relevant results	AVG TT ng/ml
1985	Häkkinen	Serum hormones during prolonged training of neuromuscular performance	RT group	24w 3d.w	RT - Periodised	NSΔ TT	↑ BM, ↑ MIF, ↓ BF% TT/SHBG & MVC TT/CORT & MVC	7.46 7.41
			PWR group	24w 3d.w	PWR	↑ TT weeks 0-8, 0-16, NSΔ 0-24	↑ MIF, ↓ BF% TT/SHBG & MVC	8.33 8.5
1987	Häkkinen	Relationships between training volume, physical perf cap, serum hormone concentrations during prolonged training in elite weightlifters	(~7y TE)	1 y	OLY	NSΔ TT ↑ LH at 4,6,8,12m	↓ TT during 2-week preparatory phase	5.13 6.37
1988	Häkkinen	Neuromuscular and hormonal adaptations in athletes to strength training in two years	(~7y TE)	2 y	OLY	↑ TT and LH 0-24m	↑ TG, FFW, OLY results, SJH NSΔ MIF 0-24m	5.37 7.23
2003	Ahtiainen	Muscle hypertrophy, hormonal adaptations and strength development during strength training in strength-trained and untrained men	(BB, WL)	21w 2d.w	RT mixed	↑ TT, FT 0-14 ↓ TT, FT 14-21	TT & MVC (R=0.84)	NR NR

Adaptation in testosterone from chronic resistance training

The clinical range for normal testosterone levels is not consistent across different laboratories across the globe. However, traditional reference ranges for "clinically normal" levels are from about 2.75 – 10 ng/ml, with some laboratories reporting a minimum of 1.7 ng/ml and some reporting as high values as 17.27 ng/ml (Bhasin et al., 2008). Clinical secondary hypogonadism is defined as <1.5 ng/ml (Bhasin et al., 2010), at which point men are placed on replacement therapy until they reach the 3 – 7 ng/ml range. A commonality in the resistance-trained literature is the relative basal testosterone levels observed in these men. The range of testosterone levels (means) observed in resistance trained populations ranges from 5.13 ng/ml (Häkkinen et al., 1987) to 11.96 ng/ml (Willoughby et al., 2014). Three of these studies displayed testosterone values above the 70th percentile of the normal range (2.75-10 ng/ml). In novice individuals generally testosterone levels are much lower, 3.69 – 8.46 ng/ml, with all studies in the 50th percentile for the normal range (2.75-10 ng/ml) (see Table 1). Looking at the data in this manner suggests that over time testosterone levels increase in men that continuously perform resistance exercise. It is reasonable to believe that this increase in testosterone levels is a chronic adaptation of the HPG axis, which occurs to maximise gains within a resistance trained population. As a person's training age increases their relative gains in strength and hypertrophy progressively decrease, until they plateau at their genetic ceiling. It is plausible that if this adaptive increase in testosterone did not occur then, this plateau might be realised sooner, thus reducing their potential for training gains. The importance of higher resting testosterone levels in appears to provide improvements in strength, power and

hypertrophy gained from resistance training. This effect is augmented in research investigating increased testosterone levels beyond clinical ranges.

Effect of supraphysiologic levels

The relative importance of testosterone-mediated gains becomes increasingly evident when exogenous supplementation is used to boost levels beyond normal clinical ranges. Increase in testosterone from exogenous administration of 600mg testosterone enanthate ranges from 550%-750%, which is considered supraphysiologic levels (Bhasin et al., 1996). This study explored four groups: exercise and placebo; exercise and testosterone; no exercise and placebo; and no exercise with testosterone. The group with the greatest benefits was the exercise combined with testosterone supplementation, observing improvements in quadriceps and triceps hypertrophy and squat strength. The testosterone-only group also saw improvements in triceps and quadriceps hypertrophy as well as increases in bench and squat strength. This suggests that exogenous testosterone supplementation markedly enhances the chance of interaction with AR, either within the muscles, causing hypertrophy (Heemers & Tindall, 2007) or interacts with neuron AR, resulting in strength improvements (Fraley & Ulibarri, 2002; Hammond et al., 2001; Herbst & Bhasin, 2004). A further mechanism of these marked improvements is the upregulation of satellite cell activity (Sinha-Hikim et al., 2003). Despite these obvious improvements from anabolic steroids, the relative importance of testosterone in normal and resistance trained populations is still not clear. Moreover, the beneficial effects of testosterone boosters, like d-aspartic acid, (which may increase testosterone within normal clinical ranges) is not completely clarified. Data suggests that higher basal levels may be important in a trained population. If d-aspartic acid can increase testosterone within trained

individuals, then noticeable increases in strength and or hypertrophy might be experienced.

D-Aspartic acid supplementation in humans

Research in the supplementation of d-aspartic acid within humans is still in its infancy. To date, only six DAA supplement studies have been conducted within a human population (Table 3). The earliest study carried out in humans was in 2009 (Topo et al., 2009). This initial experiment involved a group of IVF patients (n=23) who ingested a solution of 10ml of 2.0 M sodium D-aspartate (3.12g). The placebo group ingested a solution of 2 M NaCl (1.12g). After 12 days of supplementation levels of luteinizing hormone and testosterone were significantly increased by 33.3% and 42% respectively. At three days' post suspension testosterone was still significantly increased compared to baseline (11.5%) (Topo et al., 2009). Of note was the subject's original levels of total testosterone (TT) at 4.5±0.6 ng/ml which, inclining towards low-normal levels of testosterone, potentially attributed to the relatively sharp increase in total testosterone that was observed. D-aspartic acid has been shown to improve sperm quality in subfertile men (IVF patients), affected by various levels of rapid progressive spermatozoa mortality and low total concentrations (D'Aniello et al., 2012). In this study, a solution of 10ml of 2.0 M sodium (2.66g DAA) was supplemented daily for 90 days. Improvements in a total concentration of spermatozoa and motility were observed. The authors reported similar increases in LH and TT as Topo, 2009, at 1.3–1.6 fold, however as these values were not published, the baseline TT levels are not clear. These two studies suggest that one of DAA's functions is to help regulate normal testosterone levels.

Table 3 D-aspartic acid research to date conducted in human males. Abbreviations and symbols within the table: *in vitro* fertilization (IVF), no training (NA), total testosterone (TT), luteinizing hormone (LH), training age (T), non-significant change (NSΔ), fat-free mass (FFM), muscle strength (STR), gonadotropin releasing hormone (GnRH), estradiol (E2), body mass (BM), bench press (BP), muscle endurance (END), sleep (SLP), physical appearance (APP), * research conducted in products similar to DAA or containing DAA as a blend, # of note, average (AVG).

Year	1st Author	Name	Population information	Study Length	Training	Hormones	Other relevant results	AVG TT ng/ml
2009	Topo	The role and molecular mechanism of DAA in the release and synthesis of LH and TT in humans and rats	IVF patients	12d	NA	↑ TT, LH	TT ↑ for three days' post	4.50 6.40
2012	D'Aniello	d-Aspartate, a key element for the improvement of sperm quality	Infertile men	3m	NA	↑ TT, LH		Not reported
2013	Willoughby	DAA combined with 28 days RT has no effect on body comp, muscle strength, serum hormones associated with HPG axis in RT men	T > 1y	28d 3d.w	RT periodised	NSΔ TT	↑ FFM, ↑ STR (both groups)	8.08 8.88
2014	Willoughby *	RT and NMDA has no effect on body Comp, muscle performance, and serum hormones in RT males	T > 1y	28d	RT periodised	NSΔ TT, LH, GnRH, E_2	↑ FFM, ↑ BM, ↑ STR (both groups)	11.96 12.02
2015	Bloomer *	Influence of a DAA/Na NO_3/Vitamin D3 supplement on physiological parameters in middle-aged men – pilot study	Overweight/ Obese men ~42y	28d	NA	NSΔ TT (↑ TT in some with low baseline)	↑ vitality, libido, energy levels	2.60 (3g) 2.90 2.90 (6g) 3.10
2016	Rodgers *	Impact of a multi-component supplement on testosterone, nitrate/nitrite and physical performance in RT men	T > 0.5y	28d	Normal RT, No formal program	NSΔ TT # p>0.050, *d*=0.648	↑ 5-Set BP test ↑ perception of STR, END, SLP & APP	6.2 5.1

In 2013 a study combined weight training (4d.w) with supplementation (3g DAA) in resistance trained men (Willoughby & Leutholtz, 2013). They observed similar increases in hypertrophy (fat-free mass) and 1RM strength (leg press) between groups, yet no differences in total testosterone (8.08±0.69, 8.88±0.5 ng/ml, DAA from day 0-29 respectively) or free testosterone were observed. As previously highlighted, extended resistance training results in a gradual increase in testosterone, which is evident in the baseline levels of these participants. Perhaps with resistance training, there is a natural upregulation of DAA which is negating any potential benefit from exogenous supplementation. Alternatively, with testosterone levels near the upper limit of normal ranges, negative feedback mechanisms within the HPG axis might downregulate testosterone production regardless of any possible accumulation of DAA within tissues such as the anterior pituitary or testes (A. D'Aniello et al., 1996). Willoughby et al. also measured serum levels of DAA and DAA oxidase (DDO), which is an enzyme known to degrade DAA. They noted no group differences in serum DAA, but significant increases in serum DDO. This suggests an alternative hypothesis whereby increased DDO activity is degrading DAA faster than it can accumulate within target tissues (Willoughby & Leutholtz, 2013). In 2014 these same authors explored the effects of a proprietary blend product involving the methylated form of DAA (NMDA), DAA, trimethylglycine and S-adenosyl methionine (SAMe) (Willoughby et al., 2014). The manufacturer's theory (no data published) of this blend, was to upregulate NMDA receptors within the body to activate the HPG axis (see Figure 4 on page 16), to increase the output of LH and TT. The results from this study also showed similar hypertrophy and strength gains across the study population, with no group interaction effects for TT (11.96±1.26 to 12.02±1.18 ng/ml, experimental

group means from days 0-29 respectively), FT or LH. Similar to the previous research, baseline levels of testosterone observed in this study are relatively high, and thus previously mentioned homeostasis negative feedback mechanisms could be preventing any further potential increase. Recently the effects of another proprietary blend was explored in resistance trained men (Rodgers et al., 2016). The supplement D-Pol™ containing 3.12g of DAA, sodium nitrate (480mg) and Vitamin D3 (4000IU) was given to 24 resistance trained men, subjects had a history of weight training, with no break from training in the last six months before the study. No significant changes were observed in hormones tested, including total testosterone (6.2±2.2, 5.1±1.2 ng/ml, d-aspartic acid means from day 0-29 respectively). The authors observed an improvement in a five-set bench press challenge. However, they believed this might have been attributed to the sodium nitrate portion of the blend, rather than DAA. This highlights an issue that can arise in supplement research that involve blends, introducing confounding variables that make it difficult to draw solid conclusions about one ingredient over another. As more research is explored with pure DAA over proprietary blends, and we begin to understand the effects of DAA within humans better, the value of this research will increase.

It is evident that d-aspartic acid research within humans is still in its infancy, and more research is required. Currently, the only positive effects of supplementation have been observed in men with infertility issues and relatively low baseline testosterone. Furthermore, no advantageous strength or hypertrophy adaptations have been found in conjunction with resistance training. To date, there is no data investigating dosage in a resistance trained population, where higher levels may be required to see an effect. As resistance trained populations adapt more slowly than

novice individuals, an extended training study is required to delineate if time is an important factor with the supplementation of d-aspartic acid in this population.

Summing argument

Since the mediating factors for the relationship between testosterone and strength are still unclear, further research into this area is needed. Past research indicates that d-aspartic acid can elevate basal testosterone levels in infertile men. However, the research in resistance trained populations is not as promising. Additional research is still required, as the literature has yet to explore higher dosages of the supplement, which may be a requirement in a resistance trained population. Furthermore, d-aspartic acid has a plausible mechanism to directly affect the nervous system due to its potential role as a neurotransmitter. The proposed research will further elucidate the efficacy of d-aspartic acid on improving training outcomes from a three-month periodised training bout.

CHAPTER 3 – DOSING STUDY METHODS

Subjects

The institutional review board approved the study and participants provided written informed consent prior to testing and participation. A total of twenty-four participants from the local area completed this study. To be eligible participants had to be: male; aged 18–36; have no acute or chronic medical conditions; have the ability to bench press 100% body weight, and had been performing regular resistance training exercise for at least three days per week for the previous two years. No participants were supplementing their diet with any ergogenic or testosterone boosting supplements prior to testing. All participants provided written consent and completed a medical history check.

Experimental approach to the problem

This was a randomised, double-blinded, and placebo-controlled design to examine the effects of d-aspartic acid supplementation on basal testosterone levels following a two-week supplementation protocol. Participants were assigned to one of three experimental groups: placebo (D0), three grams of DAA (D3) and six grams of DAA (D6). All participants consumed ten opaque capsules each morning with breakfast for two weeks. They contained either: six grams of flour (D0, n=8); a mixture of three grams each of flour and DAA (D3, n=8); or six grams of DAA (D6, n=8). Participants were randomly allocated to treatment groups following a block randomisation procedure based on a computer-generated list of random numbers. Placebo (D0), mixed (D3) and pure supplement (D6) was provided in

identical opaque capsules to improve blinding. Group allocation was managed by a technical officer, while investigators were kept blind to group assignment throughout the intervention. All participants followed an upper/lower body split resistance training program for a full month, with the initial two weeks of training (washout period) performed without supplementation. Three time points were used to obtain testing data: T1, T2 and T3 (Figure 8).

Experimental procedures

Testing sessions consisted of a fasted blood draw, then 1RM bench press evaluation. Initial baseline blood measures were taken at two time points (T1 & T2) and averaged to ensure accuracy in baseline assessment of these markers (Figure 8). After T1 prescribed training commenced for four weeks. After testing session T2 daily supplementation begun with training continuing as before. Post-measures were taken after these last two weeks of training and supplementation, at the end of week 4 (Figure 8). The supplemental period of two weeks was chosen as this has been previously shown to be a sufficient period to see a change in total testosterone levels (Topo et al., 2009).

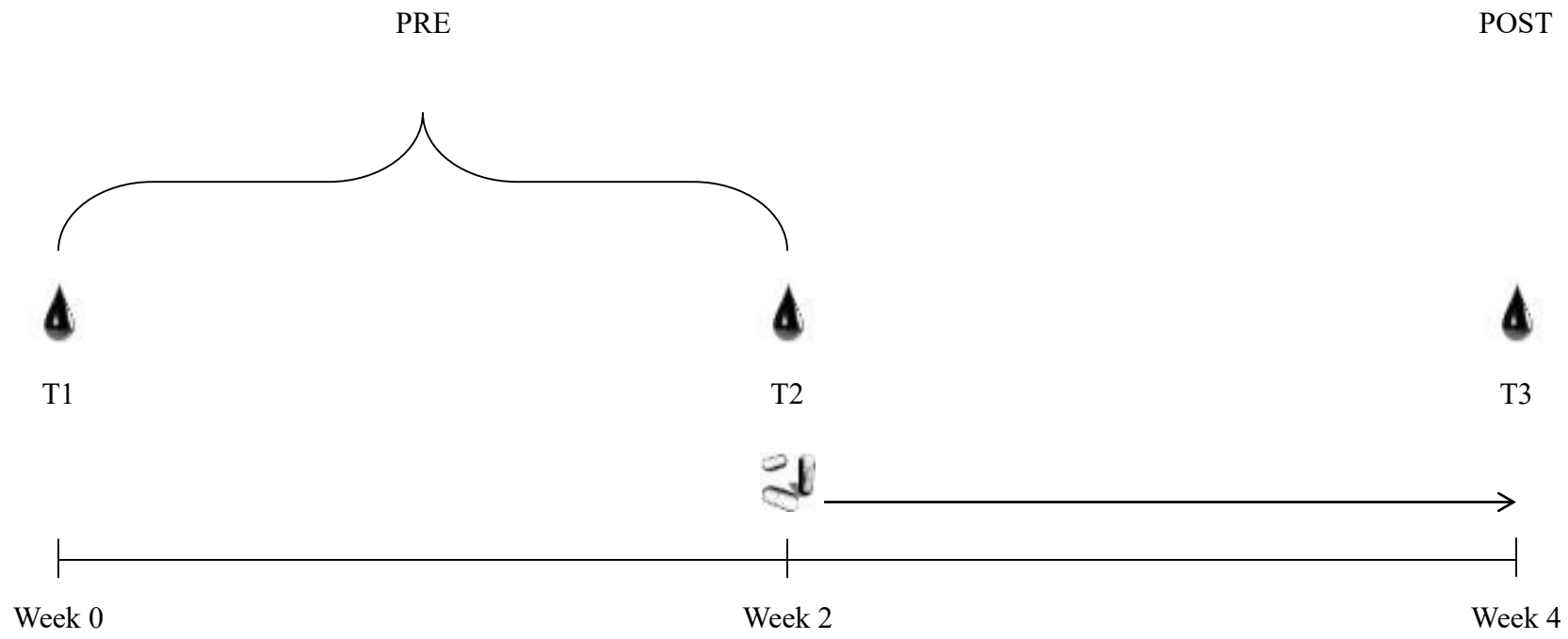


Figure 8 Timeline of the Study 1 design. Key: testing session 1 (T1), testing session 2 (T2), Testing session 3 (T3), training 4 $d.w^{-1}$ ( ), daily supplementation ( ), blood draw ( ).

1RM testing

Bench Press dynamic strength one repetition max (1RM) was measured before the standardisation period (T1), beginning of the experimental period (T2) and post-experiment period (T3) (Figure 8), as part of eligibility testing. The correct form included depth to the level of the chest, with feet not leaving the floor, and the backside is not leaving the bench at any point during the repetition. The protocol for 1RM testing involved one warm up set of 10 reps at approximately 50% of their estimated 1RM, followed by two more warm-ups at approximately 70% and 80% with only 1–2 reps. After the warm-ups, participants attempted 1RMs with incrementally increasing weight. The weight achieved before the failed attempt was recorded as the 1RM. A participant's 1RM was reached within five attempts, and adequate rest between attempts adhered to (3–5 mins) (Marshall, McEwen, & Robbins, 2011).

Fasted blood draws

All blood draws were obtained via venepuncture of the antecubital vein after a 12 hour fast. Participants were also instructed to avoid strenuous exercise and alcohol consumption the day before the draw. Blood draws were performed by a trained phlebotomist (primary investigator), and subsequent draws were planned for the same time of morning (7:00–10:00 am) for each particular participant, to prevent any effect of diurnal variation. Whole blood was collected using serum separator tubes (SST™ II Advance, BD Vacutainer®). They were then allowed to clot for 45 minutes and centrifuged using a fixed angle rotor centrifuge: ADAMS® Compact

II Centrifuge, V:227 (Becton Dickinson & Co) (828 x g, at 2700 rpm) for 15 minutes in an air-conditioned room (19 ℃). Serum was aliquoted and stored at –80 ℃ until analysis (Douglas Hanly Moir Pathology, Macquarie Park, NSW, Australia). A single analysis of serum was conducted for total testosterone, estradiol, sex-hormone-binding-globulin (SHBG) and albumin. Testosterone and SHBG was measured via electrochemiluminescence immunoassay, on a Roche E170 system (Roche Diagnostics, Dee Why, NSW, Australia), with a limit of detection values of 0.025 ng/ml and 0.350 nmol/l respectively. Albumin was measured via bromocresol green succinate buffer method, on an Abbott ARCHITECT c16000 (Abbott Laboratories, Abbott Park, Illinois, USA). The limit of detection of the albumin BCG assay is 30 g/l. Estradiol was measured via chemiluminescent microparticle immunoassay on an Abbott i2000SR (Abbott Laboratories, Abbott Park, Illinois, USA), the analytical sensitivity of the estradiol assay is ≤ ten pg/ml. Free testosterone was calculated from total testosterone, SHBG and albumin (Vermeulen, Verdonck, & Kaufman, 1999).

Training standardisation

Participants were trained for four days per week over a one-month period. The prescribed training for each exercise consisted of four sets of a repetition-maximum range of 8-10. If the repetition range was not met, participants were asked to adjust the weight accordingly in the next session. Exercises during the upper body session were: barbell bench press; overhand pulldown; barbell overhead press and underhand pulldown. The lower body session consisted of back squat; good morning; leg extensions; and straight leg calf raises. Adherence was monitored via training diaries and supervised sessions (min 1x per week).

Dietary intake

Participants were asked to control their diet, by avoiding any major changes in eating habits throughout the study. To monitor their diet, they were asked to weigh and recorded their food intake for three days each of the first and last week. Monitoring included two training days and one non-training day and these three days were averaged to get a daily mean for week one and week four. The food diaries were entered into CalorieKing (Australian Edition 4.0), then analysed for daily intakes (protein, carbohydrates and fats) and normalised to body weight.

Statistical Analysis

Analyses were conducted using IBM SPSS Statistics version 21.0 (Armonk, NY: IBM Corp), and the level of significance was set at $p<0.05$. Data are shown as mean±S.D. The distribution was tested for normality using the Kolmogorov-Smirnov test. Paired sample statistics were run on total testosterone (TT), free testosterone (FT), estradiol (E_2), sex-hormone-binding-globulin (SHBG), and albumin (ALB) to determine the stability of these blood measures over the standardisation period. As these measures were found to be unchanged, they were each computed (averaged) into one baseline measure. Univariate analysis of the absolute change scores: POST= $\left(T3 - \frac{T1+T2}{2}\right)$ was conducted, with the baseline scores: PRE=$\left(\frac{T1+T2}{2}\right)$ as covariates (Figure 8). Pairwise comparisons with Bonferroni correction were performed if a group effect was observed. To explore the responsiveness of the supplement, linear regression analysis was conducted on the baseline and change scores of TT and FT, of the experimental groups (n=16).

CHAPTER 4 – DOSING STUDY RESULTS

Analysis of the POST values revealed no main effect for group with E_2 (p=0.469), SHBG (p=0.070) and ALB (p=0.319). Post values of D6 TT were significantly reduced (~11.5±16.9 %) as compared to the pre-values (p=0.032; 5.85 to 5.12 ng/ml). FT in group D6 was significantly decreased (429.13 to 363.38 pmol/l) as compared to D0 (439.62 to 480.87 pmol/l) (p=0.005) but not D3 (534.9 to 524.3 pmol/l) (p=0.062) (Figure 9). Diet analysis revealed no significant changes in macronutrient (CHO: p=0.699; PRO: p=0.990; FAT: p=0.537) and caloric intakes (p=0.640) during the study. Regression analysis revealed no significant relationship between baseline total testosterone levels and total testosterone change (R=0.009, p=0.659), and no significant relationship between baseline free testosterone and free testosterone change (R=0.103, p=0.127).

Table 4 Participant demographics

	Placebo (*n*=8)	3 g/d (*n*=8)	6 g/d (*n*=8)
Age (years)	24.24 ± 2.26	23.16 ± 2.16	26.06 ± 4.26
Training age, (yrs)	2.94 ± 0.78	3.25 ± 1.04	4.00 ± 1.91
Height (m)	1.84 ± 0.03	1.74 ± 0.07	1.78 ± 0.06
Body Mass (kg)	89.41 ± 3.59	79.50 ± 6.07	85.12 ± 7.95
1 RM Bench (kg)	111.56 ± 15.17	97.50 ± 12.82	106.86 ± 15.74

Data are mean±SD

Table 5 Hormonal markers. PRE (Baseline), POST (T3), and Change Scores (Δ) of hormonal markers. PRE values are an average of T1 and T2.

	Total Testosterone (ng/ml)		
Time	Placebo	3 g/d	6 g/d
PRE	6.03 ± 1.48	6.95 ± 1.44	5.85 ± 1.10
POST	6.07 ± 1.35	6.91 ± 1.71	5.12 ± 1.16
Δ	0.05 ± 0.80	-0.03 ± 0.68	-0.74 ± 0.95*
	Free Testosterone (pmol/l)		
	Placebo	3 g/d	6 g/d
PRE	439.62 ± 132.64	534.88 ± 127.65	429.13 ± 93.98
POST	480.87 ± 133.48	524.25 ± 101.67	363.38 ± 78.09
Δ	41.25 ± 52.48	-10.63 ± 66.31	-65.75 ± 79.25 *
	Estradiol (pmol/l)		
	Placebo	3 g/d	6 g/d
PRE	118.50 ± 20.91	117.56 ± 30.58	107.50 ± 24.22
POST	125.12 ± 23.88	112.5 ± 34.51	104.75 ± 34.03
Δ	6.63 ± 14.94	-5.06 ± 19.52	-2.75 ± 23.46
	SHBG Pre (nmol/l)		
	Placebo	3 g/d	6 g/d
PRE	34.56 ± 16.55	32.56 ± 10.72	33.56 ± 11.82
POST	30.38 ± 12.39	32.88 ± 12.53	33.75 ± 10.98
Δ	-4.19 ± 5.90	0.31 ± 4.29	0.19 ± 1.46
	Albumin (g/l)		
	Placebo	3 g/d	6 g/d
PRE	46.38 ± 2.08	45.06 ± 2.60	45.50 ± 1.49
POST	44.75 ± 1.67	45.00 ± 2.33	45.50 ± 2.56
Δ	-1.63 ± 1.33	-0.06 ± 1.82	0.00 ± 2.35

Data is presented as: mean±SD. * statistically significant (P<0.05)

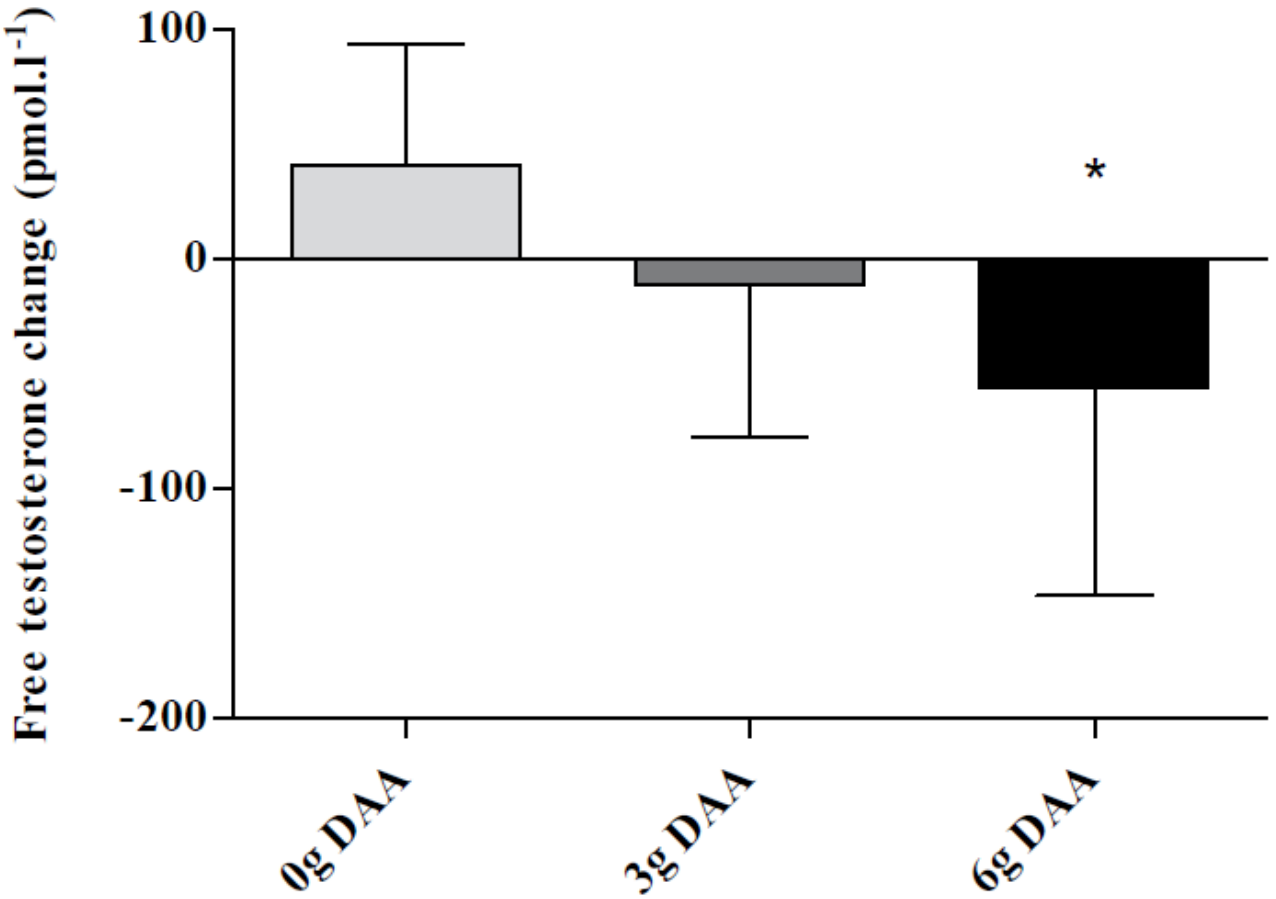


Figure 9 The absolute change of free testosterone. * statistically significant (P<0.05)

CHAPTER 5 – DOSING STUDY DISCUSSION

The aim of this dosing study was to investigate the effectiveness of three grams and six grams on raising testosterone levels in resistance trained men. The critical findings of the dosing study were, 1) resistance trained men consuming a daily dose of six grams of d-aspartic acid demonstrated significant reductions in total and free testosterone after 14 days of d-aspartic acid supplementation, and 2) the responsiveness to d-aspartic acid supplementation was unaffected by initial testosterone levels (total or free) in resistance trained men.

The present results of Study 1 demonstrate that in resistance trained men three grams daily of d-aspartic acid had no significant effect on total testosterone, estradiol, sex-hormone-binding-globulin, and albumin. This is contrary to the evidence provided by Topo et al. (Topo et al., 2009), where the experimental group consumed the same dose over 12 days and reported elevated total testosterone levels (~42 %). Baseline testosterone levels of the current study were higher than values found in Topo et al. (Topo et al., 2009) (6.3 and 4.5 ng/ml respectively), presumably because the population in the Topo et al. study was sedentary (Topo et al., 2009). In resistance training literature, total testosterone levels range from 5.8–8.6 ng/ml (Häkkinen et al., 1988a; Häkkinen, Pakarinen, et al., 1985) for trained individuals and 4.9–6.6 ng/ml for untrained (McCall et al., 1999; Reaburn et al., 1997; Staron et al., 1994). The increase in testosterone observed in Topo et al. (Topo et al., 2009) was likely because testosterone levels were low enough for d-aspartic acid to have an effect. In comparison results in the D3 group mirror the results seen in the study by Willoughby & Leutholtz (Willoughby & Leutholtz, 2013), where the total testosterone levels fall

within levels observed in resistance trained males (Häkkinen et al., 1988a; Häkkinen, Pakarinen, et al., 1985).

It was observed in the six-gram group that total testosterone was significantly reduced from baseline by ~12.5%, with a parallel decrease in free testosterone ~15.3% (see Table 5). Previous research has demonstrated that in resistance trained men, free testosterone can increase due to training (Ahtiainen et al., 2003). A reduction in calculated free testosterone in this study is due to a decrease in total testosterone, an increase in the binding proteins or a combination of the two occurring. Within the context of increasing total testosterone, a maximum effective dosage (MED) is observed in rat studies (A. D'Aniello et al., 1996). At the higher dosages, there was significantly increased accumulation of d-aspartic acid, noted in the pituitary and testes (A. D'Aniello et al., 1996). A dose response increase in total testosterone was observed until 1 µmol/g. Each increase in dose past 1 µmol/g the rise in testosterone was reduced (A. D'Aniello et al., 1996). It could be theorised that 6 g/d may be affecting negative feedback mechanisms of the HPG axis, thus reducing pituitary initiated production of luteinizing hormone and in turn testosterone levels. Furthermore, d-aspartic acid could also be over-accumulating within the testes. This over accumulation may be creating a disruptive effect on the mobilisation of cholesterol from the outer membrane to the inner (Furuchi & Homma, 2005), which would attenuate testosterone production. As this was the first study to administer a six-gram dosage of d-aspartic acid, these mechanisms can only be speculated, due to the lack of data available on the utilisation of this dosage of d-aspartic acid in humans.

The reductions in testosterone observed in this study are important to consider, owing to the negative impact it could have on training gains within this population. Resistance trained men have higher levels of strength and hypertrophy compared to novice

trainers and also exhibit higher basal testosterone levels (Häkkinen et al., 1988a; Häkkinen, Pakarinen, et al., 1985; McCall et al., 1999; Reaburn et al., 1997; Staron et al., 1994; Willoughby & Leutholtz, 2013), which suggest a link between basal total testosterone levels and training-related gains. A decrease in total testosterone with a concurrent decrease in free testosterone could reduce the likelihood of interaction with androgen receptors in muscles and nerves, which would reduce the speed of testosterone initiated muscle protein synthesis (Heemers & Tindall, 2007). Over time this could translate into reduced training gains. Conversely, alterations of testosterone within normal physiological ranges may not be clinically significant. Research indicates that when total testosterone levels are observed of normal healthy ranges (4.9-8.6 ng/ml), it affects strength and hypertrophy. In the case of hypogonadism where testosterone levels are low this negatively affects strength and hypertrophy, and with the use of steroids, a positive effect is observed (Bhasin et al., 1996; Lunenfeld, Arver, Moncada, Rees, & Schulte, 2012). The changes noted in the current study reflect minor alterations on normal testosterone physiological ranges. It is currently unknown if these fluctuations are detrimental to training gains.

Limitations

A potential limitation of this research may be the study length. The results from a two-week supplementation study will only be relevant to acute usage of d-aspartic acid. The observed reduction in testosterone may rebound, or even decrease further and a longer term training study would be able to explain the effects of this supplement better. Moreover, it would be able to delineate changes in strength and or hypertrophy and observe whether d-aspartic acid affects training-related gains positively or negatively.

Conclusion

Many testosterone boosting supplements are commercially available without sufficient research to support their efficacy. The present study has demonstrated that 3 g/d of d-aspartic acid was inadequate to affect any hormonal markers and that 6 g/d significantly reduced total testosterone and free testosterone levels, with no concurrent change in other hormones tested. It is currently unknown if any adverse impact of this reduction, on strength and hypertrophy, will occur over time. The need for longer-duration research utilising six grams of d-aspartic acid is evident. Future research should explore supplementation of 6 g/d over a longer period and observe any correlations between basal testosterone levels and changes in hypertrophy and strength.

CHAPTER 6 – TRAINING STUDY METHODS

Subjects

The institutional review board approved the study and participants provided written informed consent prior to testing and participation. To be eligible participants had to be: male; aged 18–36; have no acute or chronic medical conditions; have the ability to bench press 100% body weight, and had been performing regular resistance training exercise for at least three days per week for the previous two years. None of the participants was supplementing their diet with any ergogenic or testosterone boosting supplements before testing. All participants provided written consent and completed a medical history check during the recruitment process. Study 1 of this thesis was used to determine the sample size of the training study (Melville, Siegler, & Marshall, 2015). Free testosterone change in the six-gram group demonstrated an effect size of 1.17, resulting in a total sample size of 12 participants. To adequately power this study the number was inflated to 20 participants to accommodate for a 60% adherence rate. Of the 22 recruited subjects, three dropped out due to personal reasons, leaving 19 that completed the study. Participant demographics are presented in Table 6. The study was conducted according to the Declaration of Helsinki and was approved by the university ethics committee.

Table 6 Participant demographics

	Placebo (n=9)	6 g/d (n =10)
Age (years)	25.4 ± 6.4	22.4 ± 2.6
Training age (years)	3.3 ± 1.7	3.1 ± 1.3
Height (m)	178.3 ± 6.2	180.4 ± 6.4
Body Mass (kg)	82.5 ± 9.0	80.5 ± 10.2

Data are mean±SD. No differences were observed between PLA and DAA at T1.

Experimental approach to the problem

Study 2 was a randomised, double-blinded, and placebo-controlled study design, with the objective to examine the effects of d-aspartic acid supplementation on hormonal levels, hypertrophy, neural and strength changes during a three-month resistance training program. Participants were assigned to one of two experimental groups: placebo (PLA, 6g rice flour, n=9) or d-aspartic acid (6g DAA, n=10). All participants consumed ten identical opaque capsules each morning with breakfast for 12 weeks. Participants were randomly allocated to treatment groups following a block randomisation procedure based on a computer-generated list of random numbers. Group allocation was managed by a technical officer, while the primary investigator was kept blind to group assignment throughout the experimental intervention, and data -analysis. All participants followed a body-split periodised training program for three months' duration (4 d/w). Three time points were used to obtain all testing data, baseline (T1), six weeks' post (T2) and 12 weeks' post (T3).

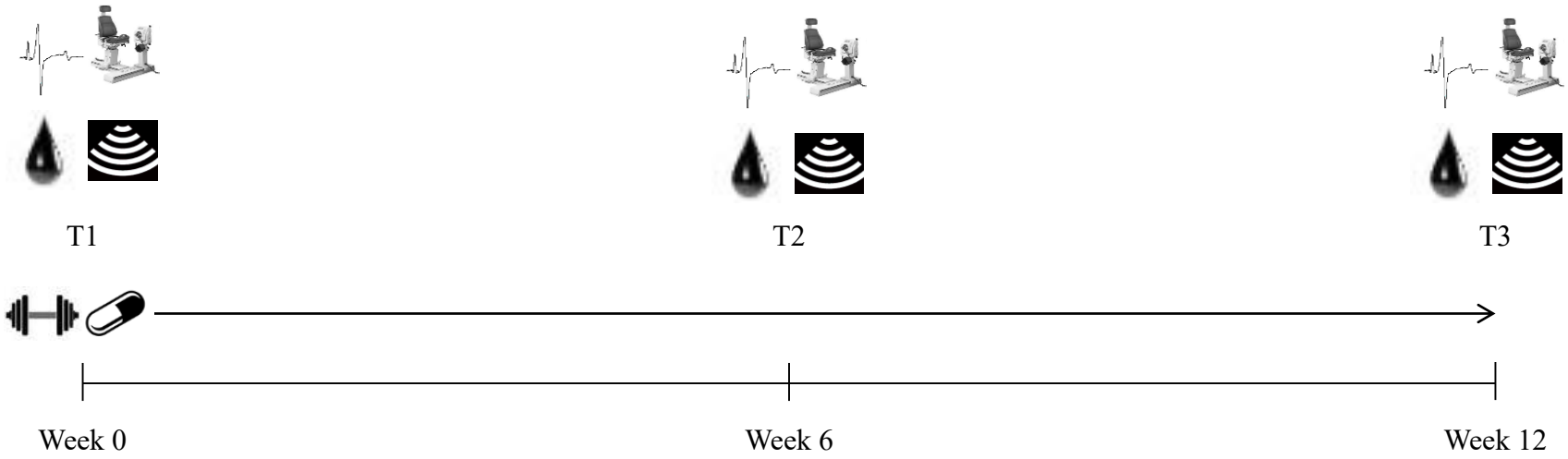


Figure 10 Timeline of Study 2 design and testing procedures. Baseline testing (T1), midpoint testing (T2), post testing (T3), electrical stimulation protocol ( ), isometric force testing of the calf and quadriceps muscles ( ), venous blood drawn and centrifuged for serum ( ), ultrasound testing of the calf and quadriceps muscle ( ), resistance training began after all testing procedures were completed for T1 ( ), supplementation protocol began after all testing procedures were completed for T1 ( ).

Experimental procedures

Testing sessions (T1-T3) involved: a fasted blood draw for hormonal and lipid measures; ultrasound measurements of the quadriceps and calf muscles (panorama and sagittal images) for hypertrophy; electrical stimulation of the calf muscles for h-curve, m-curve and v-wave neuromuscular parameters; and maximal voluntary contraction (MVC) for isometric strength of the quadriceps and calf muscles (Figure 10). Following T1, prescribed training and supplementation commenced for six weeks. The program incorporated periodisation changes, implemented from T2 (see Table 7 and Table 8). The purpose of the periodisation was to improve adherence, motivation and prevent overtraining. All participants had to follow the prescribed RT program. Post-measures were taken after another six weeks, at the end of week 12.

Training Program

Participants were trained for four days per week over the three-month period, with supervised sessions conducted once per week. The prescribed training involved five exercises that included 3-5 sets of various repetition maximum (RM) prescriptions (2RM – 10RM). A main lift was prescribed for each day – deadlift, bench press, good morning/stiff-leg deadlift and squat, with accessory exercises for balance and volume. Briefly the program was as follows: Monday focused on the hamstrings, glutes, quadriceps, trapezius, latissimus dorsi and calves; Tuesday was pectoralis, deltoids, triceps and back extensors work; Wednesday – rest day; Thursday, hamstrings, soleus, trapezius, biceps and latissimus dorsi and Friday was heavily focused on quadriceps work with some glutes and hamstrings, see Table 7 and Table 8. These changes did

not affect the general layout of the program. Training diaries were given to participants at the end of T1 to help monitor adherence rate. All participants were asked to perform 48 sessions in the gym in total, the average number of sessions was 47.53±0.84, or an adherence rate of 99.01%. Ten repetition-maximum (10RM), of the calf raise and leg extension, were also calculated from training diaries from the average weight of the sets they performed at week 1 (T1), week 7 (T2) and week 12 (T3).

Table 7 Program design from weeks 1-6. Prescribed training was either repetition maximum (RM) or body weight to failure (BWF). Exercise identifiers as they appear in the table: barbell (BB), conventional (C), machine (M), overhead (OH), stiff-leg (SL), bent-over (BO), extension (EXT), dumbbell (DB)

Weeks 1-6											
	Set 1	Set 2	Set 3	Set 4	Set 5		Set 1	Set 2	Set 3	Set 4	Set 5
Day 1						Day 3					
Deadlift (BB, C)	8RM	6RM	5RM	4RM	5RM	Deadlift (BB, SL)	10RM	10RM	10RM	8RM	
Split squats (BB)	10RM	8RM	6RM			Seated calf raise	10RM	10RM	8RM	8RM	6RM
Seated Row (M)	8RM	6RM	4RM	4RM		Row (BO, BB)	10RM	10RM	10RM		
Stiff-Arm (M)	10RM	10RM	10RM	10RM		Biceps curl	10RM	10RM	10RM	10RM	
1-leg calf raises	BWF	BWF	BWF			Chin-ups (N)	10RM	8RM	6RM		
Day 2						Day 4					
Bench Press (BB)	8RM	6RM	5RM	4RM	5RM	Front squat (BB)	8RM	6RM	5RM		
Press (OH, BB)	10RM	8RM	6RM	6RM	6RM	Leg press (M)	8RM	6RM	5RM		
Flys (DB)	10RM	10RM	10RM	10RM		Leg EXT (M)	10RM	10RM	10RM	10RM	
Dips	8RM	6RM	4RM	4RM		Leg curl (M)	10RM	10RM	10RM	10RM	
Back Extensions	10RM	10RM	10RM			Step-ups (DB)	10RM	10RM	10RM		

Table 8 Program design from weeks 7-12. Prescribed training was either repetition maximum (RM) or body weight to failure (BWF). Exercise identifiers: barbell (BB), sumo (S), split-squats (SS), machine (M), dumbbell (DB), ezy-curl bar (Ez), overhand grip (OG), 1-arm rows were prescribed at a heavy weight (8RM-10RM), continually alternated each side, until prescribed number of reps were completed (*), extension (EXT).

Weeks 7-12											
Day1	Set 1	Set 2	Set 3	Set 4	Set 5	Day3	Set 1	Set 2	Set 3	Set 4	Set 5
Deadlift (BB, S)	4RM	4RM	3RM	3RM	2RM	Deadlift (BB, SL)	8RM	8RM	6RM	6RM	
Bulgarian SS (BB)	8RM	6RM	6RM			Pull-up (OG)	8RM	8RM	6RM		
Seated Row (M)	6RM	6RM	4RM	4RM		Seated calf raise	10RM	8RM	8RM	6RM	6RM
Lat pulldown (M)	10RM	10RM	8RM	8RM		Biceps curl	10RM	10RM	10RM	10RM	
1-leg calf raises	BWF	BWF	BWF			1-arm Rows (DB)	40R	*			
Day2						Day 4					
Bench press (BB)	8RM	6RM	6RM	4RM	4RM	Back squat (BB)	8RM	8RM	6RM	6RM	
Seated press (DB)	6RM	6RM	6RM	6RM		Leg press (M)	10RM	6RM	8RM	6RM	6RM
Incline press (BB)	8RM	8RM	8RM	8RM		Leg EXT (M)	10RM	10RM	10RM	8RM	
Skullcrushers (Ez)	8RM	8RM	6RM	4RM		Leg curl (M)	8RM	8RM	8RM	8RM	
Back Extensions	8RM	8RM	8RM			Step-ups (DB)	5RM	5RM	5RM	5RM	

Fasted blood draws

All blood draws were obtained via venepuncture of the antecubital vein after a 12 hour fast. Participants were also instructed to avoid strenuous exercise and alcohol consumption the day before the draw. Blood draws were conducted by the primary investigator who was trained in phlebotomy. Subsequent time point draws were planned for the same time of morning for each particular participant, to prevent any effect of diurnal variation (maximum range 7:00–10:00 am). Whole blood was collected using serum separator tubes (SST™ II Advance, BD Vacutainer®). They were then allowed to clot for 45 minutes and centrifuged using a fixed angle rotor centrifuge: ADAMS® Compact II Centrifuge, V:227 (Becton Dickinson & Co) (828 x g, at 2700 rpm) for 15 minutes in an air-conditioned room (19 ℃). Serum was aliquoted and stored at –80 ℃ until analysis (Douglas Hanly Moir Pathology, Macquarie Park, NSW, Australia). A single analysis of serum was performed for total testosterone, estradiol, sex-hormone-binding-globulin (SHBG) and albumin. Testosterone and SHBG were measured via electrochemiluminescence immunoassay, on a Roche E170 system (Roche Diagnostics, Dee Why, NSW, Australia), with a limit of detection values of 0.025 ng/ml and 0.350 nmol/l respectively. Albumin was measured via bromocresol green succinate buffer method, on an Abbott ARCHITECT c16000 (Abbott Laboratories, Abbott Park, Illinois, USA). The limit of detection of the albumin BCG assay is 30 g/l. Estradiol was measured via chemiluminescent microparticle immunoassay, on an Abbott i2000 (Abbott Laboratories, Abbott Park, Illinois, USA) and the analytical sensitivity of this assay is ≤ 10pg/ml. Free testosterone was calculated from total testosterone, SHBG and albumin (Vermeulen et al., 1999). Whole blood was also collected in EDTA tubes with the order of collection

as EDTA then SST. After inverting the EDTA tubes, they were spun for 10 minutes (828 x g, at 2700 rpm), in an air-conditioned room (19 ℃). EDTA was aliquoted and analysed immediately for HDL levels via the dry-chemistry method on a Roche Reflotron machine (Roche Diagnostics, Dee Why, NSW, Australia). Previous research has demonstrated that the intra-assay precision of this test is below 3.2 % (Riesen, 1990).

Ultrasound

Participants were asked to rest in a supine position for 20 minutes, to allow for fluid shift to stabilise within the muscles (Berg, Tedner, & Tesch, 1993). Extended-field-of-view (EFOV) images were conducted at the lower third of the quadriceps muscles, measured as a third of the distance between the centre of the knee joint and the ASIS. This site was determined in pilot testing to be optimal to observe the cross-sectional area of the quadriceps muscles. B-mode ultrasound imaging was conducted using an Echo Blaster 128 family scanner, Echo Wave II v2.3.6 (Telemed, Vilnius, Lithuania) software. EFOV imaging is created by scanning over the muscle, with new image frames combining with the previous frames to form a panorama image (Noorkoiv, Nosaka, & Blazevich, 2010). These images were analysed for CSA of the quadriceps muscles; vastus lateralis (VL_C), vastus intermedialis (VI_C), vastus medialis (VM_C) and rectus femoris (RF_C). Incorrect estimation of the quadriceps muscle can occur if the image is not scanned perpendicular to the muscle. To prevent this effect, indelible ink was used to mark the scan line. While adhering to this scan line, the probe had to be moved continuously, held at a 90-degree angle to the skin using as light pressure as required to keep the probe in contact with the skin. These factors, along with visual inspection of the image were used to control the quality of the final images, with scans

repeated where necessary. A minimum of three images was obtained for each time point, with the average values from these images used for data analysis. Images were analysed using ImageJ 1.46 (National Institutes of Health, Bethesda, Maryland, USA) public domain software package. Intra-experimenter reliability (CV) of the EFOV method was 2.26%.

Sagittal images were obtained again at the inferior third of the quadriceps, and at the midpoint of the quadriceps (50% between the centre of the knee joint and ASIS). The probe was manipulated at the marked site until the superficial, and deep aponeuroses of vastus lateralis/fat layer and vastus lateralis/vastus intermedialis layers were parallel, and the pennate fibres were straight as opposed to curved (Figure 11). Three images were obtained at these two sites for all time points. Muscle thickness was obtained for vastus lateralis ($VL_{T30,}$ VL_{T50}) and vastus intermedialis ($VI_{T30,}$ VI_{T50}) at the midpoint of the image, with two extra measures equal distance from the middle. Pennation angle was determined three times for vastus lateralis (VL_A), at the midpoint quadriceps site (50% between the centre of the knee joint and ASIS). Final data points were averaged across the multiple images and measures. For the calf muscle, only muscle thickness of the soleus and gastrocnemius muscles was obtained. The distance between the lateral malleolus and the fibular head was divided into three sites: Superior quarter, SOL_{Th75} and GAS_{Th75}; superior third, SOL_{Th67} and GAS_{Th67}; and midpoint, SOL_{Th50} and GAS_{Th50}. The average values around the centre of the image for the three images were used for data analysis. Intra-experimenter reliability (CV) of the analysis of the sagittal images was 2.99% for the quadriceps thickness, 8.68% for calf thickness and 13.67 % for pennation angle.

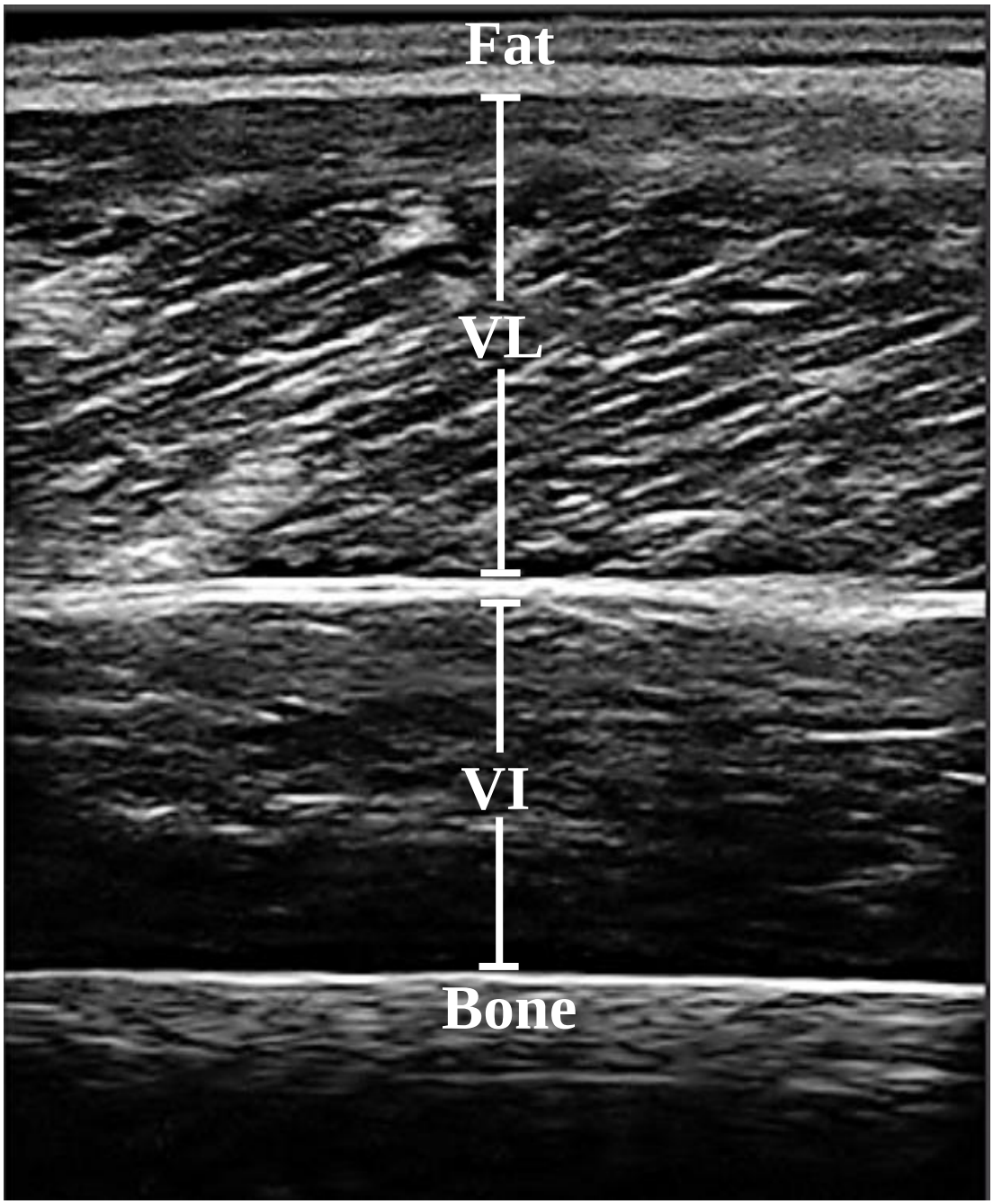


Figure 11 Typical ultrasound sagittal image of the left lateral quadriceps. Features from top to bottom of the image are: fat layer, aponeurosis, vastus lateralis, aponeurosis, vastus intermedialis and femur.

Nerve stimulation and isometric strength

EMG

Surface electromyography (sEMG) was recorded from the soleus and gastrocnemius muscles using bipolar Ag/AgCl electrodes (Maxensor; Medimax Global, Australia). Electrodes (10mm contact diameter, 10mm inter-electrode distance) were placed parallel to the muscle fibres following careful skin preparation (removal of excess hair, skin abrasion, cleaning the skin with isopropyl alcohol swabs) to reduce the skin electrical impedance to below 5 kΩ. Soleus electrodes were placed at two-thirds of the distance between the medial condyle of the femur and the medial malleolus and for medial gastrocnemius, the two electrodes were put on the muscle belly. For each participant anatomical landmarks were recorded in relation to the placement of the electrodes to ensure consistency between testing sessions. A reference electrode was placed on the medial malleolus. An ML138 Octal BioAmp (common mode rejection ratio >85 dB @ 50 Hz, input impedance 200 MΩ) with 16-bit analog-to-digital conversion, sampled at 2 kHz (ADI Instruments, Sydney, NSW, Australia) was used to record sEMG signals into LabChart v7.3.7. Raw signals were filtered with a fourth-order Bessel filter between 20-500 Hz.

Posterior tibial nerve stimulation

Transcutaneous stimulation of the tibial nerve was applied to the left knee. The cathode, a single Ag/AgCl electrode (Maxensor; Medimax Global, Australia), was located on the posterior side of the knee within the popliteal fossa. The exact location was identified by manipulating a rubber insulated portable cathode around the

popliteal fossa while stimulating at a low intensity until the largest evoked resting H-wave was elicited from soleus. The anode was created from a sheet of aluminium foil (Al, 10x6 cm) and electrode paste (Ten20®, Weaver and Company, Colorado, USA). This was applied to the anterior aspect of the thigh just proximal to the patella. H-reflexes, M-waves and V-waves were evoked from the soleus and gastrocnemius muscles using a 1-ms square wave pulse delivered by a constant current stimulator (DS7AH Stimulator, Welwyn Garden City, UK) applied at 400 V.

Isometric Force

Maximal calf muscle strength was measured as maximal isometric calf flexion torque exerted in an isokinetic dynamometer (KinCom 125, version 5.32, Chattanooga, TN, USA). The device was configured so the seat was set upright, with the participant's hip and knee flexed to 90o degrees and the subject's lateral malleolus in line with the centre of rotation of the lever arm. The seat angle was raised slightly so that there was no gap between the knee and the edge of the seat to prevent unwanted muscle activity. The subject was strapped in with one seatbelt running across the knees and another running from left shoulder to the right hip. This setup was found in pilot testing to reduce the contribution from the hip and thigh muscles. Subjects were warmed up with three submaximal contractions (~50%, ~75%, ~90% MVC) spaced one minute apart. After sufficient warm up, three maximal voluntary contractions (MVCs) of the calf muscle were recorded, with two minutes' rest given between each MVC. Torque output signals were directly sampled from the dynamometer at 2 kHz (PowerLab, ADInstruments, Sydney, NSW, Australia). Following MVCs was the determination of H-reflex threshold and the maximal motor response. The participant was asked to hold a 10% contraction during all stimulation events. Adherence was helped by normalising

the level of MVC to 100% and then providing a monitor with a duplicate display of LabChart. This monitor displayed an enlarged mini-window, with up-to-date force information, and participants were asked to watch this window to maintain their goal of 10% tonic contraction. H-wave threshold (H_{th}) was approximated by increasing stimulation from 10 to 30 mA, by 1 mA per pass. The beginning of the H-M curve was defined as 3 mA lower than the stimulation intensity at which H-wave was ≥ 1 mV. Stimulation was then increased in multiples of 10 mA until Mmax was determined, which was defined as the last stimulation in a plateau spanning over three stimulation points. Between these two stimulation intensities (H_{th} – M_{max}), 30 points were created on a logarithmic scale (Vila-Cha et al., 2012). The H-M curve was then mapped to the thirty points with two stimulations performed at each intensity (10s rest between each stimulation). M-wave and H-waves were calculated from the peak-to-peak amplitudes of the first and second evoked waves (~10 ms and ~35 ms respectively), following the stimulation artefact (Figure 12). After the H-M curve mapping, the M_{max} intensity was used to determine the supramaximal stimulation intensity (130% M_{max}). The subjects were then asked to perform three MVCs of approximately five seconds duration, with two minutes rest in between. Along the plateau of the MVCs, a supramaximal stimulus was applied to the tibial nerve (manually triggered) to elicit a V-wave (Figure 13)

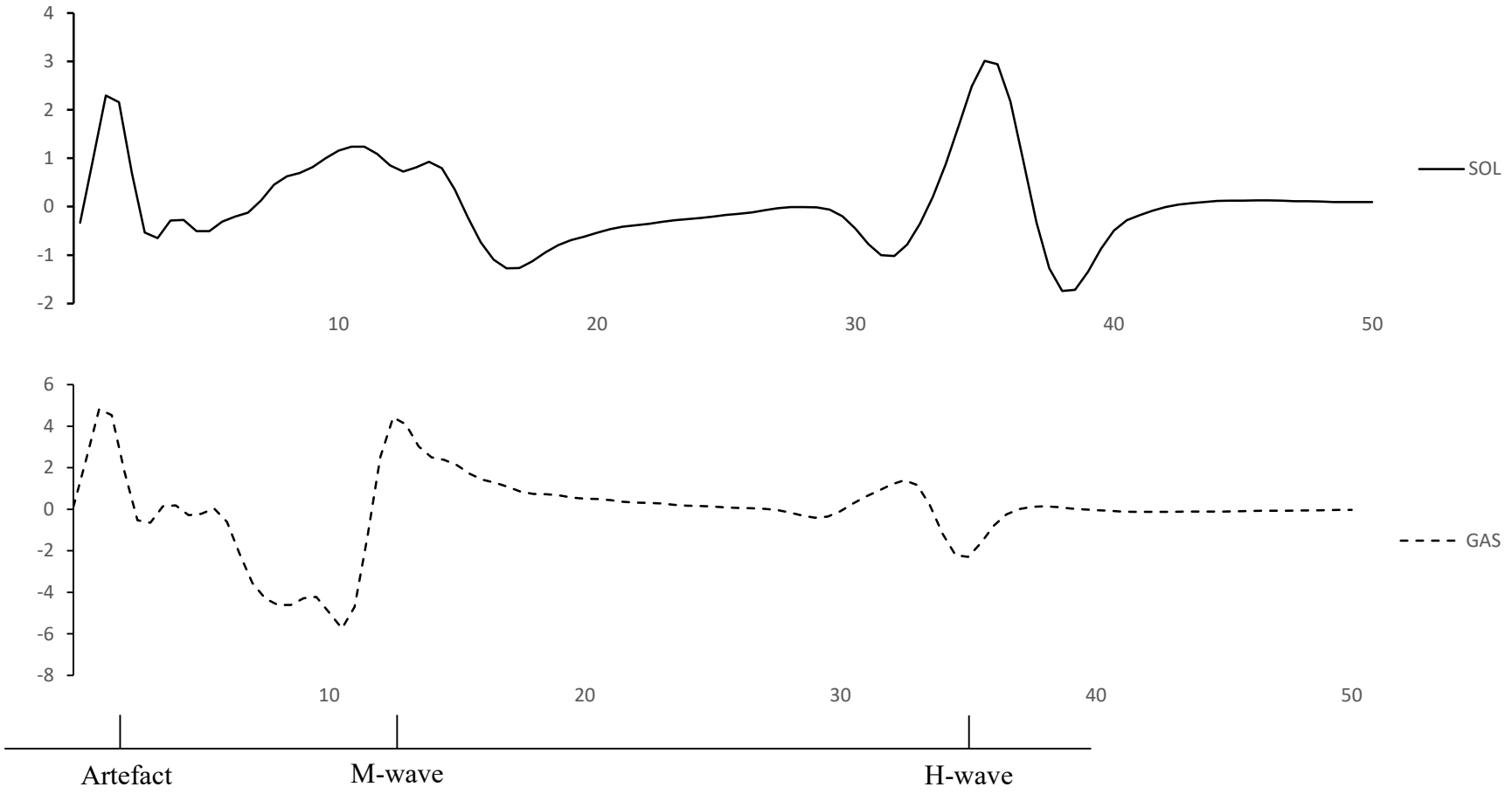


Figure 12 EMG trace of soleus. This example is at the H_{max} intensity (0-50ms), with soleus on the top trace and gastrocnemius muscle on the bottom trace.

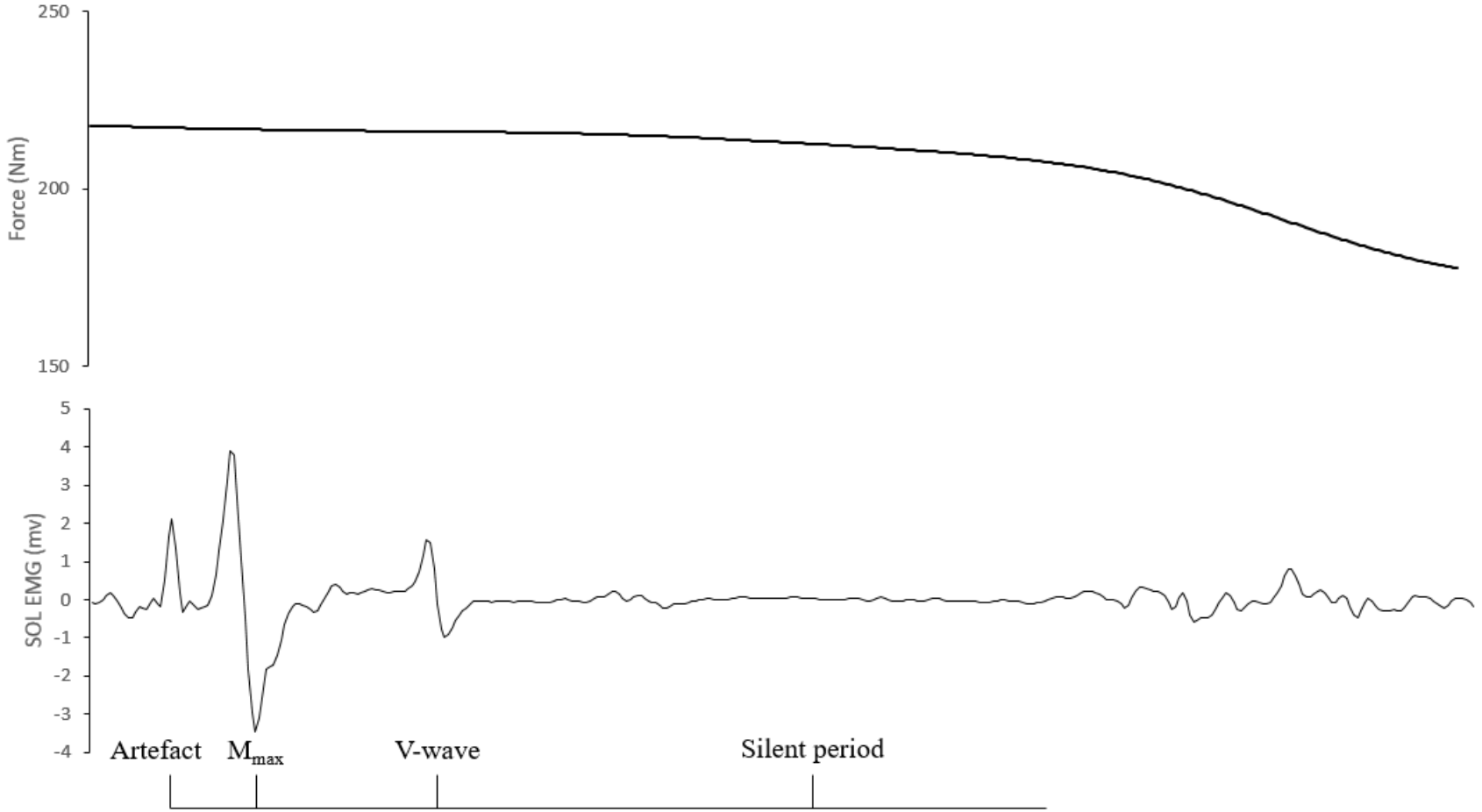


Figure 13 Zoomed figure of V-wave data. Force trace portrays delayed effect of stimulation. Soleus EMG trace depicts maximum motor response wave (M_{max}) and the elicited volitional wave (V-wave).

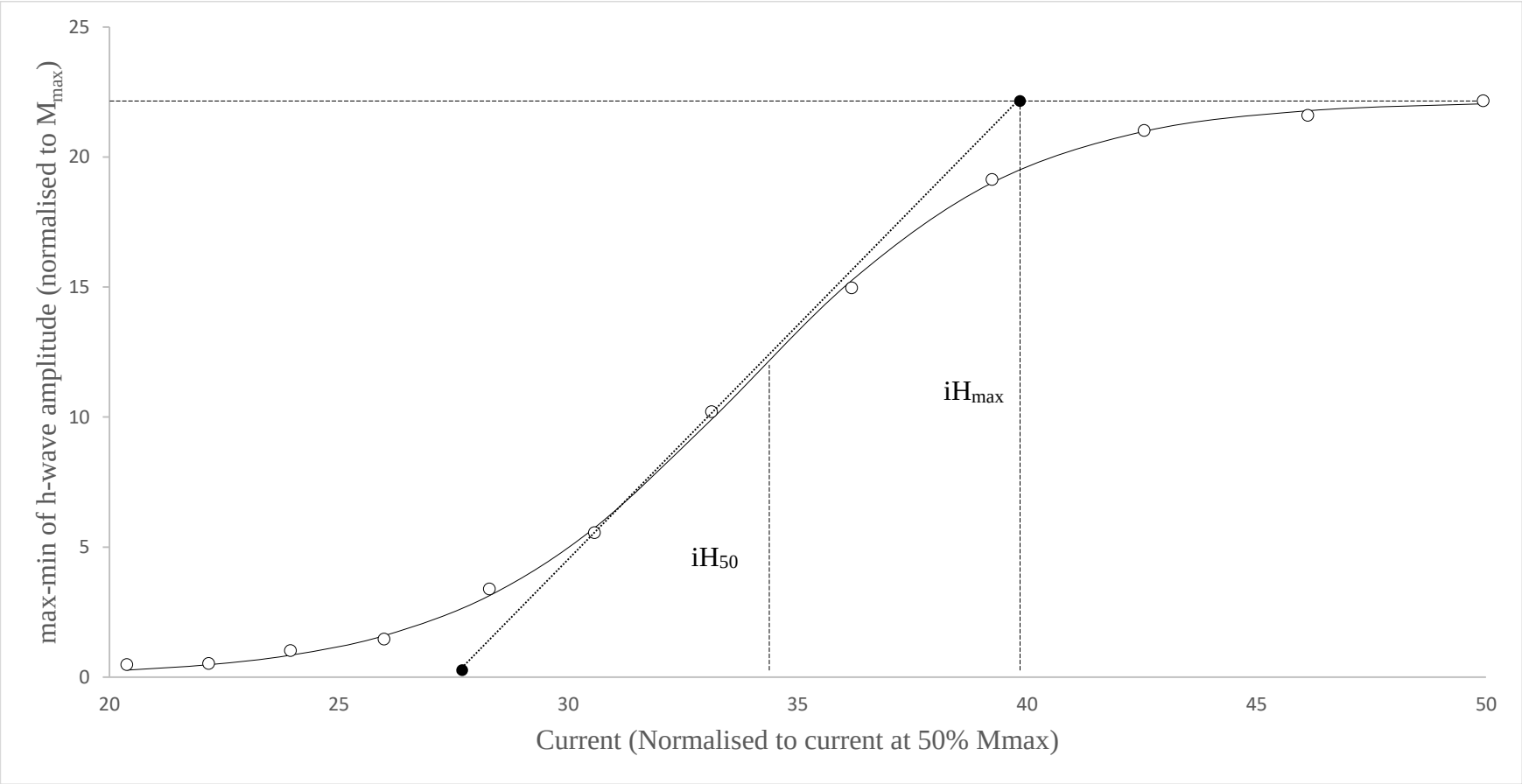


Figure 14 H-reflex parameters displayed on a representative example The raw data is represented as open circles, with the sigmoid fit data represented along the solid line. The short dashed line represents the slope at 50% of H_{max}. The lower filled circle represents $iH_{th,}$ and the upper filled circle represents iH_{max}.

MVCs for the isometric knee-extension were performed after V-wave recordings. The Kin Com was re-configured so that the participant's knees and hips were at 90^{o} degrees. The centre of their knee joint aligned with the centre of rotation and the ankle pad was fixed 2cm above the lateral malleolus. Participants were strapped in and then performed a series of warmups (~25, ~50, ~75% of their perceived maximal effort). After a short break the participants were asked to perform a maximal effort as hard and as fast as possible, after completely relaxing the lower body. Verbal encouragement was provided over the ~5s duration of the MVC. Three MVCs were recorded with two minutes' rest between each.

Force Data Processing

MVC was defined as the peak force recorded over the entire ~5s force trace. During calf stimulation protocol, if MVCs were required to be calculated from V-wave traces then the peak force was defined as maximum level excluding any superimposed twitch observed on the trace. RFD was derived as the average tangential slope of the moment-time curve (Δtorqure/Δtime), over time-windows of 0-30, 0-50, 0-100 and 0-200 ms in relation the onset of contraction. The onset of contraction was defined as a torque of 7.5 Nm above baseline levels. Maximal rate of force development (RFD_{max}) was calculated as the greatest 5-ms average slope over the entire contraction.

H-wave data processing

Peak-to-peak amplitudes of the H-wave and M-wave were calculated from the sEMG of the soleus and medial gastrocnemius. The average of the two elicited H-waves at each stimulation intensity was recorded. H_{max} amplitude was defined as the largest H-wave recorded over the H-M curve. The ascending limb was defined as data from, the initial stimulation point, to H_{max}. Before analysis, h-reflex data points were normalised to the M_{max} of that particular time point, with stimulus intensity (current) normalised to the stimulus intensity at 50% M_{max}.

Data from the ascending limb was entered into a custom program coded in the statistical package: R (R Foundation for Statistical Computing, Vienna, Austria). This program incorporated a three-parameter sigmoid function (Equation 1 below) and used a general least squares model to find the best fit for the ascending limb H-wave data (Klimstra & Zehr, 2008). Recruitment curves with an R^2<0.900 were omitted from analysis (Vila-Cha et al., 2012). The following parameters were acquired from the ascending limb: the slope at the midpoint (H_{slp}), current at H-wave threshold (iH_{th}), current at H-wave maximum amplitude (iH_{max}) and current at 50% H_{max} (iH_{50}). These parameters are visually represented in Figure 14.

$$H(s) = \frac{H_{max}}{1 + e^{m(iH_{50} - s)}}$$

Equation 1 (Klimstra & Zehr, 2008)

Slope at 50% H_{max} was estimated around H_{50} and iH_{50}, using the following differential formula:

$$\frac{Amplitude}{Stimulation\ Current}$$

$$SLP_{50} = \frac{(H2 - H1)}{(i2 - i1)}$$

Equation 2

Estimation of the slope parameter (m) for Equation 1, was determined from Equation 2 and Equation 4.

$$H_{slp} = \frac{m(H_{max})}{4}$$

Equation 3

$$m = \frac{(SLP_{50} \times 4)}{H_{max}}$$

Equation 4

H_{max} was estimated as the largest value in the h-reflex curve, and iH_{50} was estimated as the stimulus at half H_{max}. These estimations were used in Equation 1 as part of the nls(stat) function, which determined the parameters of the model: m, Hmax and iH50, where the sigmoid function best fit the data. The current intensities, iH_{max} and iH_{th}, were calculated by entering the parameters into Equation 5.

$$y = mx + b$$

Equation 5

The y-intercept (b) can be solved, as iH_{50} is determined from Equation 1 and H_{slp} is determined via Equation 3 (using m and H_{max}). If y=50% H_{max}, m=H_{slp} and x=iH_{50}:

$$b = (\frac{1}{2} \times H_{\max}) - (H_{slp} \times iH_{50})$$

Equation 6

iH_{max} can be determined from the x-intercept when y=H_{max}:

$$iH_{max} = \frac{(H_{max} - b)}{H_{slp}}$$

Equation 7

iH_{th} can be determined from the x-intercept when y=0:

$$iH_{th} = \frac{-b}{H_{slp}}$$

Equation 8

Statistical analysis

Analyses were conducted using IBM SPSS Statistics version 22.0 (SPSS Inc., Chicago, IL, USA), and statistical significance was set at $p<0.05$. The distribution was tested for normality using the Kolmogorov-Smirnov test and visual inspection of skewness and kurtosis. All measurements were analysed by a two-way (condition x time) ANOVA for repeated measures (T1, T2 and T3). In the event of a significant *F* ratio, *post hoc* comparisons were completed with a Bonferroni correction. One participant from the placebo group did not have sufficient data for calf strength and neural measures, thus was omitted from those analyses. Linear regression analysis was performed for the entire sample, to explore potential relationships between fasting hormones and training outcome variables. These analyses were investigated over three periods, T1-T2, T2-T3 and T1-T3. Unless otherwise stated, data are shown as mean±S.D.

CHAPTER 7 – TRAINING STUDY RESULTS

Hormonal analysis

There was no main effect of time for the blood markers, FT (p=0.661), SHBG (p=0.180), ALB (p=0.096), HDL (p=0.733) and a trend for a time effect for TT (p=0.075). No significant change was observed in TT across the study population, from T1-T2 (p=0.614), T2-T3 (p=0.130, d=0.317) or T1-T3 (p=0.708) (see Figure 15). A significant group by time effect (p=0.023) was observed in E_2 (Table 9), with *post hoc* analysis showing that the DAA group experienced a 16.2±14.2% reduction in E_2 from T1 to T3 (p=0.009) which was significantly different (p=0.005) to the lack of change observed in the PLA group (Figure 16). All other blood markers showed no significant group by time effect (TT, p=0.614; FT, p=0.543; SHBG, p=0.829; ALB, p=0.393; HDL, p=0.301).

Dynamic Muscle strength

No group by time effects were observed in either 10RM leg extension or 10RM calf raise (p=0.540, p=0.092, respectively). A significant main effect for time was observed in both leg extension (p<0.001) and calf raise (p<0.001). *Post hoc* analysis revealed that dynamic leg extension strength significantly increased from T1 to T2 (30.6±21.4%, p<0.001), T2 to T3 (14.4±14.7 %, p<0.01) and T1 to T3 (48.7±26.7%, p<0.001). Similarly, dynamic calf raise strength significantly increased from T1 to T2 (53.1±54.3%, p<0.001), T2 to T3 (16.0±13.0%, p<0.001) and T1 to T3 (80.0±76.5%, p<0.001).

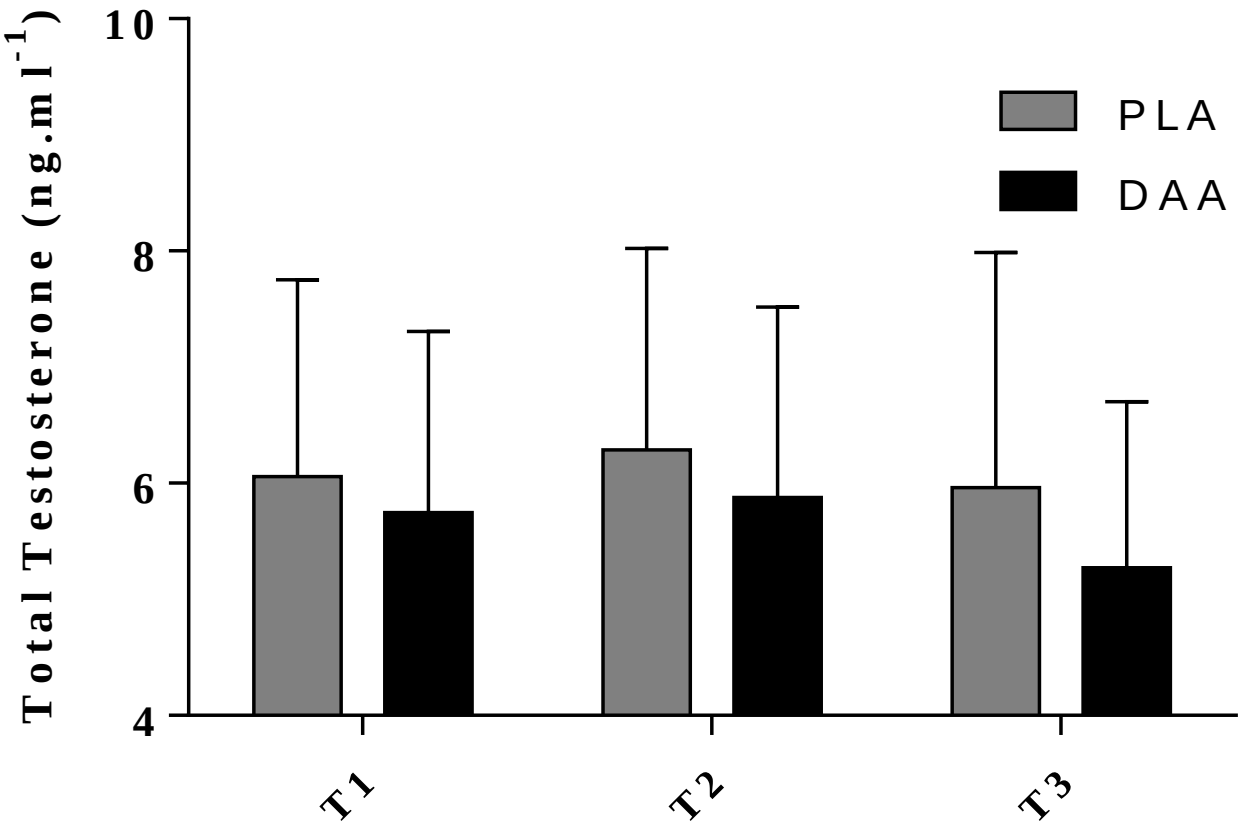


Figure 15 Total testosterone levels over the course of the 12 weeks. Data are mean±SD.

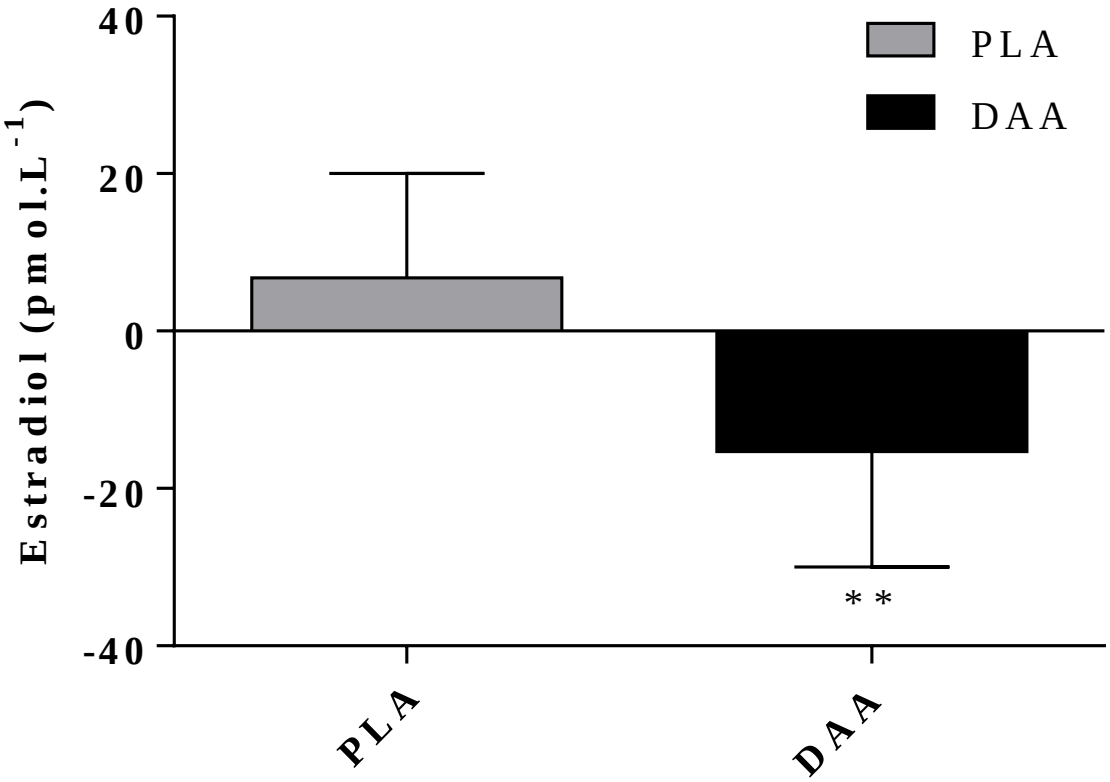


Figure 16 Absolute change score in estradiol from T1-T3. Data are mean±SD. ** significantly different from placebo ($p<0.01$).

Isometric muscle strength

No group by time effects were observed in either MVC results (calf raise, p=0.610; leg extension, p=0.585). A significant main effect for time was observed for leg extension (p=0.001), and calf raise strength (p=0.004) (Table 11). *Post hoc* analysis revealed that isometric leg extension strength increased from T1 to T2 by 10.6±14.6% (p=0.020) and also from T1 to T3 by 16.9±20.5% (p=0.011) (Figure 17). Isometric calf strength increased at T3 by 17.2±20.1% (p=0.008) (Figure 18).

Rate of force development

Group by time effects was observed in $QRFD_{25}$ (p=0.033) and $QRFD_{50}$ (p=0.038). Further analysis revealed that from T1 to T3, DAA significantly increased as compared to PLA in $QRFD_{25}$ and $QRFD_{50}$ (p=0.007, p=0.010 respectively). Specifically, from T1-T3 the PLA group did not change ($QRFD_{25}$, p=0.156; $QRFD_{50}$, p=0.319), while the DAA group experienced a 26.7±24.8% increase in $QRFD_{25}$ (p=0.024) and a 30.3±28.2% increase in $QRFD_{50}$ (p=0.025) (see Figure 19). A significant main effect for time was observed in Q_{max} (p=0.044) and Q_{200} (p=0.017) with a trend observed in Q_{100} (p=0.078). *Post hoc* analysis showed a trending increase from T1 to T3 in Q_{200} (p=0.058, d=0.625), but no significant changes in Q_{max} (p=0.123) and Q_{100} (p=0.154).

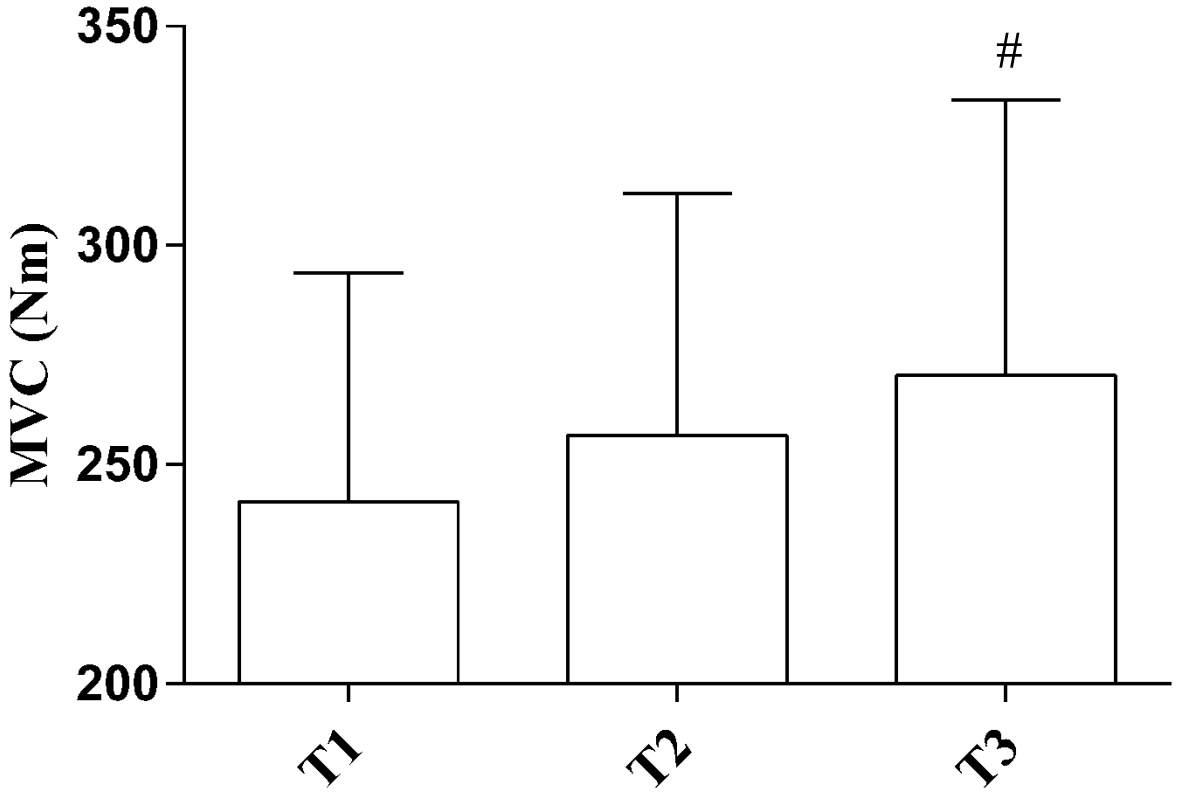


Figure 17 Maximal voluntary contraction of the isometric seated leg extension Values at baseline (T1), six-week mid-point (T2) and 12-week post testing (T3), n=19. Data are mean±SD of the pooled study sample (PLA and DAA). # significantly different from T1, irrespective of group ($p<0.05$).

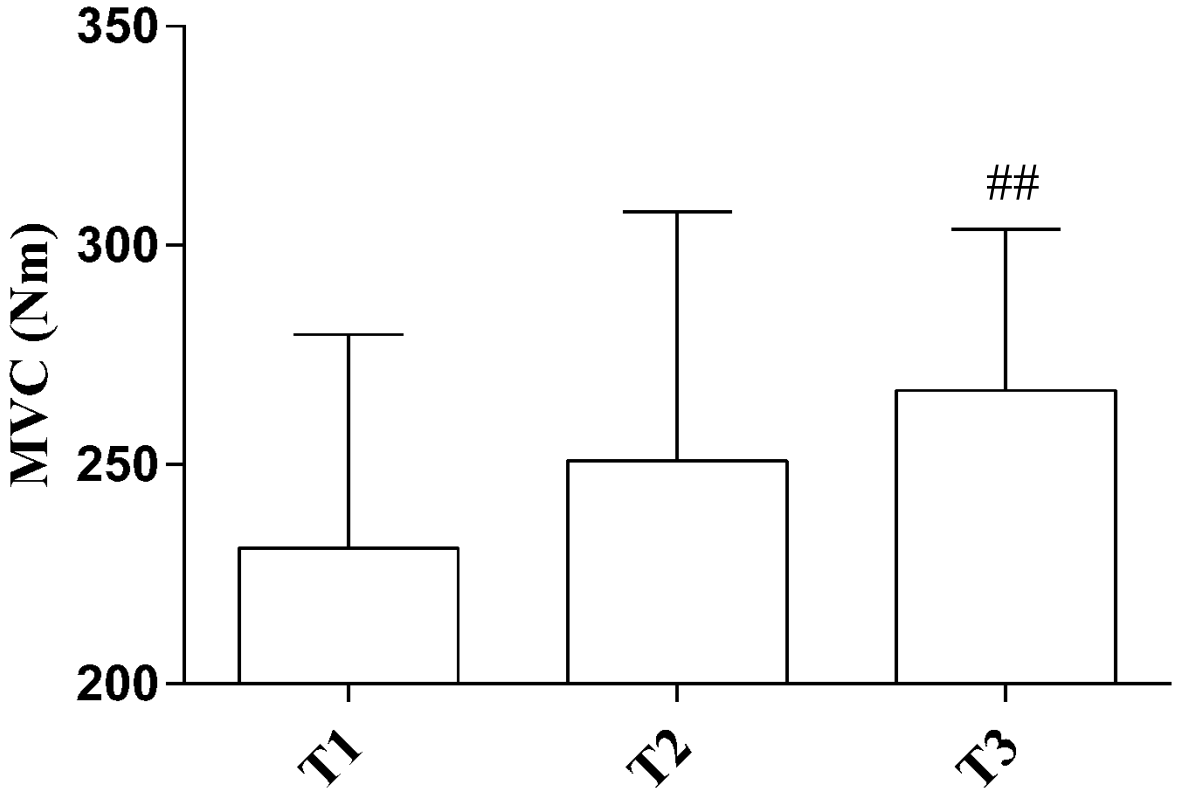


Figure 18 Maximal voluntary contraction results for isometric seated calf raise. Values at baseline (T1), six-week mid-point (T2) and 12 weeks post testing (T3), n=18. Data are mean±SD of the pooled study sample (PLA and DAA). ## significantly different from T1, irrespective of group ($p<0.01$).

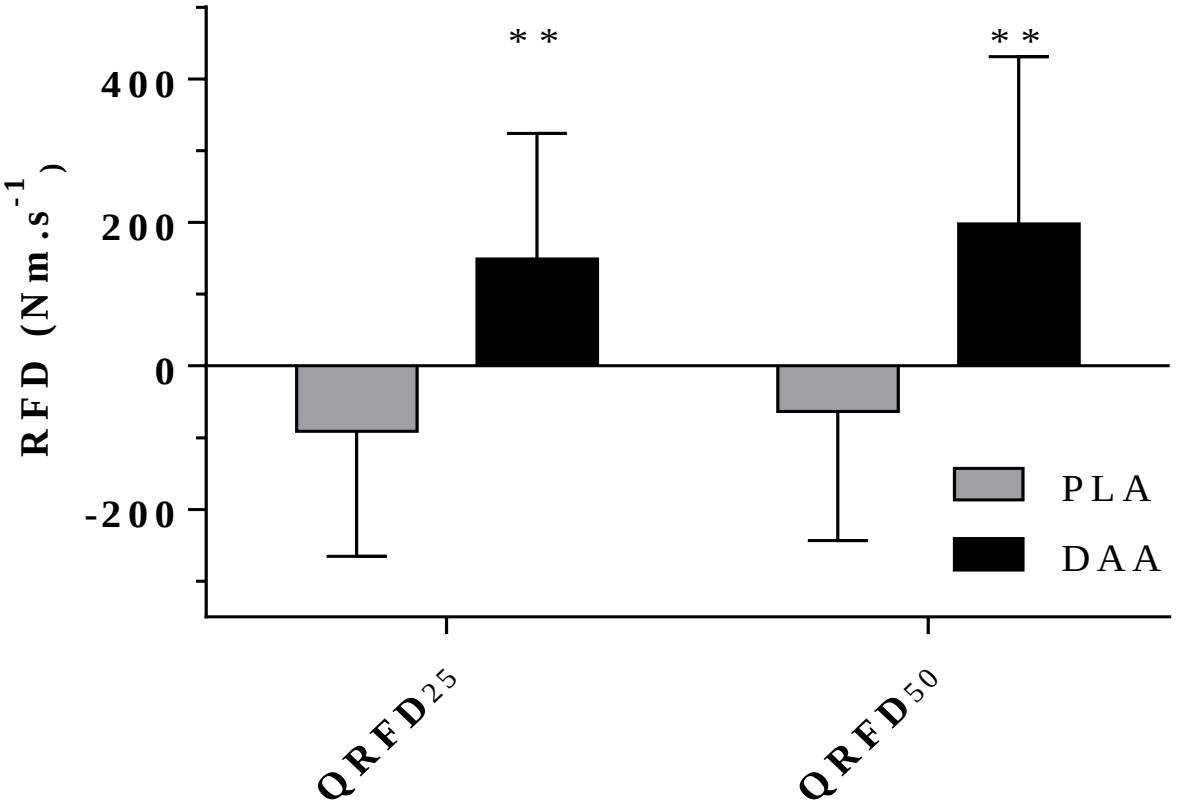


Figure 19 Absolute change score in leg extension RFD. 0-25 ms, QRFD25 and 0-50 ms, QRFD50, from baseline to post testing (T1-T3**).** Data are mean±SD. ** significantly different from placebo ($p<0.01$).

Table 9 Fasting hormones. Total testosterone (TT), free testosterone (FT), estradiol (E_2), sex-hormone-binding-globulin (SHBG), albumin (ALB) levels, for placebo (PLA) and six grams per day of d-aspartic acid (DAA), at baseline (T1), six-week mid-point (T2) and 12-week post testing (T3).

	PLA (n=9)			DAA (n =10)		
	T1	T2	T3	T1	T2	T3
TT (ng/ml)	6.1 ± 1.5	6.3 ± 1.5	6.0 ± 1.9	5.7 ± 1.6	5.9 ± 1.6	5.3 ± 1.4
FT (pmol/l)	431.9 ± 113.3	443.3 ± 121.2	444.4 ± 164.2	408.3 ± 90.3	418.7 ± 108.7	387.9 ± 95.1
E_2 (pmol/l)	84.4 ± 25.0	82.8 ± 17.7	91.2 ± 26.5	92.4 ± 25.7	83.2 ± 29.5	77.1 ± 26.4**
SHBG (nmol/l)	36.2 ± 12.5	38.2 ± 14.5	34.6 ± 11.7	34.5 ± 15.3	34.6 ± 12.8	32.3 ± 10.2
ALB (g/l)	45.4 ± 2.1	44.7 ± 3.1	44.6 ± 1.7	45.9 ± 2.5	46.1 ± 2.5	44.9 ± 1.9

Data are mean±SD. ** significant between-group difference, as compared to T1 ($p<0.01$).

Table 10 Dynamic 10 RM strength data and body mass changes in Study 2. Data extracted from training journals

	PLA (n=9)			DAA (n =10)		
	T1	T2	T3	T1	T2	T3
Leg extension	45.6 ± 19.1	58.6 ± 24.9$^{\#\#}$	62.8 ± 22.1$^{\#\#@@}$	40.3 ± 12.0	53.6 ± 17.8$^{\#\#}$	61.7 ± 19.1$^{\#\#@@}$
Calf raise	33.9 ± 7.0	41.1 ± 6.5$^{\#\#}$	47.8 ± 7.0$^{\#\#@@}$	31.3 ± 12.4	47.5 ± 10.5$^{\#\#}$	53.7 ± 9.8$^{\#\#@@}$
Body Mass (kg)	82.5 ± 9.0	83.2 ± 7.9	83.8 ± 8.0$^{\#}$	80.5 ± 10.2	81.0 ± 9.8	81.4 ± 9.9$^{\#}$

Data are mean±SD. $^{\#}$ significantly different from T1, irrespective of group ($p<0.05$), $^{\#\#}$ ($P<0.01$). $^{@@}$ significantly different from T2, irrespective of group ($p<0.01$).

Table 11 Maximal voluntary contractions and rate of force data. Seated 90^{o} isometric leg extension (Quad) and seated 90^{o} calf raise (Calf), for placebo (PLA) and six grams per day of d-aspartic acid (DAA), at baseline (T1), six-week mid-point (T2) and 12-week post testing (T3).

	PLA (n=9), PLA calf (n=8)			DAA (n =10)		
	T1	T2	T3	T1	T2	T3
Quad						
MVC (Nm)	236.0 ± 56.9	246.3 ± 61.2	261.1 ± 66.1$^{\#}$	234.6 ± 58.0	266.0 ± 50.6	278.7 ± 62.0$^{\#}$
$QRFD_{max}$(Nm/s)	1326.2 ± 527.1	1281.6 ± 497.5	1357.8 ± 483.1	1217.5 ± 444.0	1353.9 ± 355.9	1491.0 ± 281.4
$QRFD_{25}$ (ms)	846.2 ± 310.3	820.7 ± 308.6	755.1 ± 249.7**	749.3 ± 256.3	814.0 ± 303.4	898.6 ± 195.6**
$QRFD_{50}$ (ms)	1028.7 ± 423.4	1005.1 ± 387.5	965.0 ± 336.2**	922.3 ± 348.7	999.3 ± 375.2	1120.0 ± 260.2**
$QRFD_{100}$ (ms)	999.2 ± 435.2	989.3 ± 367.2	1034.1 ± 361.5	939.5 ± 358.6	996.3 ± 334.5	1098.2 ± 266.3
$QRFD_{200}$ (ms)	735.8 ± 272.0	740.0 ± 230.9	797.7 ± 253.2	705.8 ± 226.3	774.5 ± 218.7	836.1 ± 177.1
Calf						
MVC (Nm)	264.3 ± 52.0	292.8 ± 49.8	293.0 ± 40.8$^{\#\#}$	243.6 ± 61.7	268.8 ± 78.8	287.6 ± 64.8$^{\#\#}$
$CRFD_{max}$(Nm/s)	942.9 ± 246.7	992.4 ± 398.6	1094.1 ± 360.1	845.3 ± 212.3	1015.3 ± 315.4	1015.0 ± 413.2
$CRFD_{25}$ (ms)	476.0 ± 144.2	517.6 ± 227.5	487.5 ± 232.9	442.8 ± 140.8	530.3 ± 172.9	460.4 ± 212.9
$CRFD_{50}$ (ms)	588.5 ± 183.1	615.5 ± 300.1	609.1 ± 306.7	537.4 ± 172.8	663.0 ± 228.1	577.8 ± 280.3
$CRFD_{100}$ (ms)	718.7 ± 230.6	727.0 ± 372.2	757.6 ± 380.5	641.8 ± 204.7	801.6 ± 280.9	743.4 ± 348.4
$CRFD_{200}$ (ms)	653.6 ± 187.7	672.9 ± 246.9	721.3 ± 277.8	582.8 ± 135.9	712.2 ± 213.8	718.2 ± 290.5

Data are mean±SD. $^{\#}$ significantly different from T1, irrespective of group $^{\#}$ ($p<0.05$), $^{\#\#}$ ($p<0.01$). * significant between group difference, as compared to T1 ($p<0.05$), ** ($p<0.01$).

α-motor neuron excitability

H-wave, M-wave and V-wave parameters are presented in Table 12 and Table 13. A trend for a time effect was observed in gastrocnemius H_{max} (p=0.091), with no other time effects observed in the other gastrocnemius h-reflex variables (p>0.140). No significant differences for H_{max} were observed from T1 to T3 (p=0.246). A significant group by time effect was observed in gastrocnemius iH_{max} (p=0.026), and in iH_{50} (p=0.048). *Post hoc* analysis revealed that iH_{max} PLA was reduced 27.0±27.1% (p=0.048) from T1 to T3 and 25.3±20.6% (p=0.049) from T2 to T3 in PLA compared to DAA (Figure 20). *Post hoc* analysis for iH_{50} showed a between-group trend, T1 to T3 (p=0.070, *d*=0.996), and between-group significance observed from the T2 to T3 change (p=0.032) (Figure 21). PLA tended to decrease from T1 to T3 (22.6±25.6%, p=0.062 *d*=1.156) and from T2 to T3 (21.9±20.8%, p=0.081, *d*=0.836). No within-group change was observed in DAA iH_{50} from T1 to T3, or from T2 to T3. No time effects (p>0.200) or group by time effects (p>0.100) were observed in any soleus h-reflex measure.

Efferent drive and M-wave data

The V/M_{max} ratio had no main effect for time ($V/M_{max,}$ p=0.417; V/M_{max}, p=0.587, soleus and gastrocnemius respectively) or group by time effect ($V/M_{max,}$ p=0.568; V/M_{max}, p=0.496, respectively). A trend for a group by time effect was observed for soleus M_{max} (p=0.055). No significant differences were observed in soleus M_{max} (T1-T2, p=0.128; T1-T3, p=0.420; T2-T3, p=0.110). No time effect (p=0.173) or group by time effect (p=0.867) was observed for gastrocnemius M_{max}.

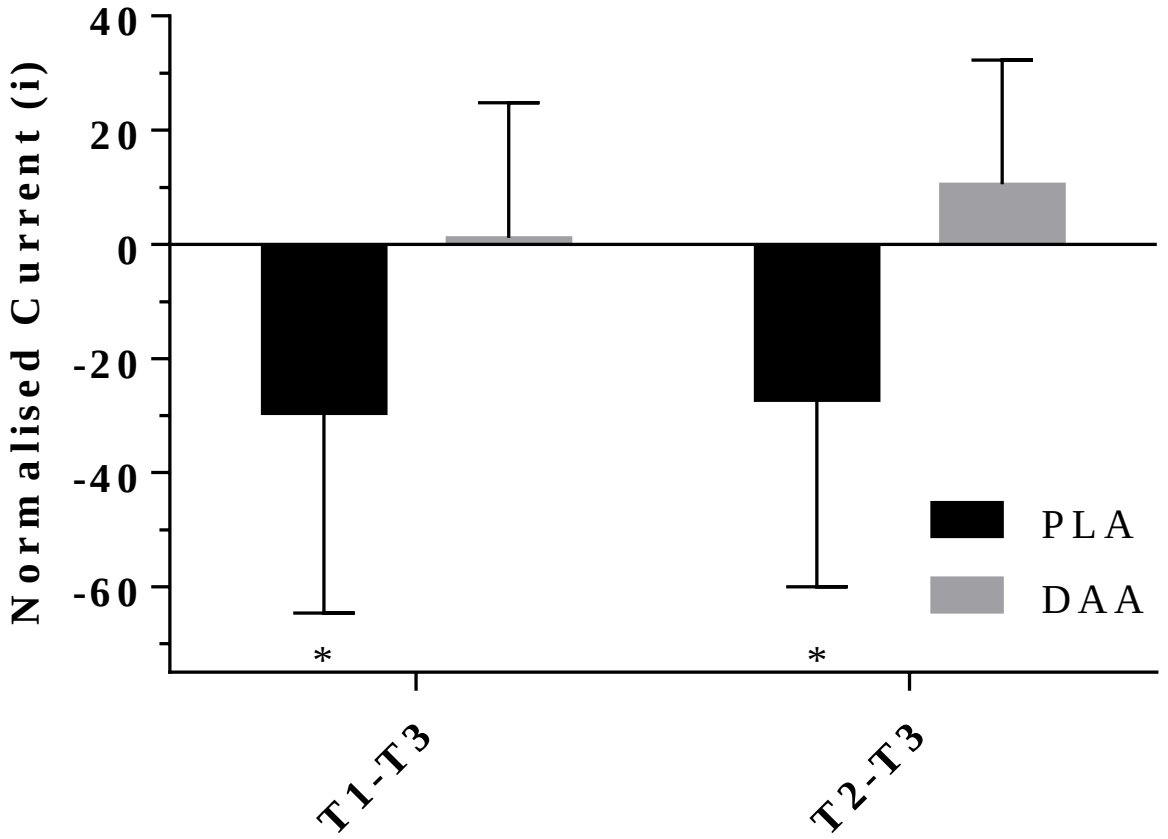


Figure 20 Absolute change score in normalised iH_{max}. (Normalised to current at 50% M_{max}), from baseline to post testing (T1-T3) and midpoint to post testing (T2-T3). Data are mean±SD. * significantly different from placebo ($p<0.05$).

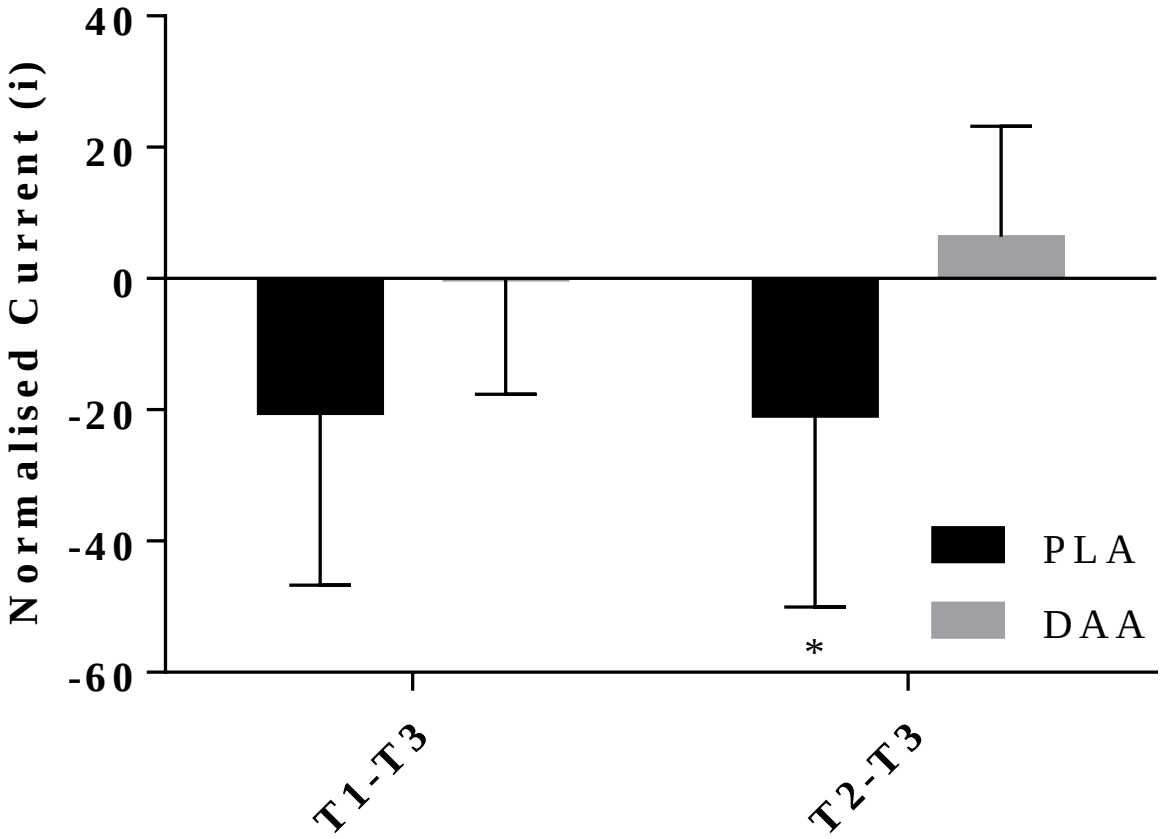


Figure 21 Absolute change score in normalised iH_{50}. (Normalised to current at 50% M_{max}), from baseline to post testing (T1-T3) and midpoint to post testing (T2-T3). Data are mean±SD. * significantly different from placebo ($p<0.05$).

Table 12 Soleus neural variables. Maximal M-wave (M_{max}), V-wave to maximal M-wave ratio (V/M_{max}), maximal H-wave (H_{max}), slope at 50% H_{max} (H_{slp}), current (i), current at H-wave threshold (iH_{th}), current at 50% H_{max} (iH_{50}), current at H_{max} (iH_{max}), for placebo (PLA) and six grams per day of d-aspartic acid (DAA), at baseline (T1), six-week mid-point (T2) and 12-week post testing (T3).

	Placebo (n=9)			6 g/d (n =10)		
Soleus	T1	T2	T3	T1	T2	T3
M_{max}, mV	9.5 ± 3.5	8.0 ± 2.3	9.8 ± 4.4	9.8 ± 2.6	11.3 ± 5.4	8.9 ± 4.0
V/M_{max}, %	30.46 ± 27.75	34.08 ± 29.28	26.49 ± 18.60	24.89 ± 9.33	30.37 ± 17.62	28.92 ± 20.29
H_{max}, % M_{max}	44.1 ± 18.3	47.4 ± 18.2	47.6 ± 15.4	45.3 ± 12.4	46.3 ± 17.4	39.8 ± 12.5
H_{slp}, mV/mA	2.1 ± 1.4	2.6 ± 2.3	2.8 ± 1.7	2.1 ± 1.3	2.9 ± 2.3	2.3 ± 1.7
iH_{th}, % i at 50% M_{max}	59.0 ± 27.0	50.8 ± 23.0	40.3 ± 8.8	47.8 ± 19.1	44.5 ± 17.1	49.8 ± 21.0
iH_{50}, % i at 50% M_{max}	70.8 ± 29.5	62.4 ± 25.6	51.0 ± 9.5	60.8 ± 23.5	54.8 ± 20.1	62.5 ± 20.6
iH_{max}, % i at 50% M_{max}	82.7 ± 32.3	74.1 ± 29.4	61.8 ± 12.5	73.8 ± 28.3	65.0 ± 23.7	75.2 ± 22.2

Data are mean ± SD.

Table 13 Gastrocnemius neural variables. Maximal M-wave (M_{max}), V-wave to maximal M-wave ratio (V/M_{max}), maximal H-wave (H_{max}), slope at 50% H_{max} (H_{slp}), current (i), current at H-wave threshold (iH_{th}), current at 50% H_{max} (iH_{50}), current at H_{max} (iH_{max}), for placebo (PLA) and six grams per day of d-aspartic acid (DAA), at baseline (T1), six-week mid-point (T2) and 12-week post testing (T3).

	Placebo (n=9)			6 g/d (n =10)		
Gastrocnemius	T1	T2	T3	T1	T2	T3
M_{max}, mV	7.7 ± 3.0	9.0 ± 3.1	8.9 ± 3.5	9.7 ± 2.8	10.1 ± 3.5	10.7 ± 2.9
V/M_{max}, %	26.50 ± 28.41	30.87 ± 28.76	24.50 ± 18.54	22.20 ± 9.91	25.30 ± 10.41	27.40 ± 30.24
H_{max}, % M_{max}	44.0 ± 26.5	35.4 ± 17.4	34.6 ± 18.9	40.7 ± 21.7	37.7 ± 17.3	29.3 ± 12.2
H_{slp}, mV/mA	1.8 ± 1.2	2.3 ± 1.6	2.9 ± 2.4	2.3 ± 1.6	2.8 ± 1.8	1.7 ± 1.2
iH_{th}, %i at 50% M_{max}	52.1 ± 18.6	55.3 ± 31.6	40.0 ± 7.6	48.9 ± 18.4	45.2 ± 17.0	47.8 ± 20.6
iH_{50}, %i at 50% M_{max}	68.2 ± 23.9	68.6 ± 34.6	47.7 ± 7.7	60.3 ± 22.6	53.7 ± 20.0	60.1 ± 22.5$^{\phi}$
iH_{max}, %i at 50% M_{max}	84.3 ± 30.9	82.0 ± 38.8	54.6 ± 8.2	71.7 ± 27.5	62.2 ± 23.3	72.8 ± 26.3*$^{\phi}$

Data are mean±SD. * significant between-group differences, as compared to T1 ($p<0.05$). $^{\phi}$ significant between-group differences, as compared to T2.

Muscle hypertrophy and architecture

Quadriceps cross-sectional area (CSA), muscle thickness and pennation angle are presented in Table 14, and calf muscle thickness is presented in Table 15. No group by time effects were observed for any of the body composition measures: body mass ($p=0.759$); quadriceps CSA ($p>0.400$); quadriceps thickness ($p>0.300$); calf thickness ($p>0.100$); quadriceps pennation angle ($p>0.300$). A significant main effect for time was observed in body mass ($p=0.005$, Table 10), with *post hoc* analysis revealing that the study population had increased by 1.5±2.3 % from T1 to T3 ($p=0.045$).

Results in the EFOV images demonstrated significant main effects for time in all CSA quadriceps measures: VL_C ($p<0.001$), VI_C ($p=0.002$), RF_C ($p=0.034$) and VM_C ($p<0.001$). *Post hoc* analysis revealed that VL_C was significantly increased from T1 at T2 (6.5±8.5%, $p=0.020$) and T3 (10.1±9.4%, $p<0.001$) and VM_C was significantly increased from T1 at T2 (7.8±7.1%, $p<0.001$) and T3 (11.4±10.0%, $p<0.001$). VI_C was significantly increased from T1 to T3 (6.6±8.7%, $p=0.008$), with a trend observed between T1 and T3 in RF_C ($p=0.091$, $d=0.573$). See Figure 22 for a representative example of the quadriceps hypertrophy that occurred.

A significant main effect for time was observed in VI_{T33} ($p=0.047$), and in pennation angle, VL_A ($p=0.040$). Other thickness variables for the quadriceps muscles did not reach significance (VL_{T50}, $p=0.059$, $d=0.198$; VL_{T33}, $p=0.055$; VI_{T50}, $p=0.676$). *Post hoc* analysis revealed a trend for an increase from T1 to T3, in VI_{T33} ($p=0.135$, $d=0.511$), VL_{T33} ($p=0.150$, $d=0.515$) and VL_A ($p=0.106$, $d=0.476$).

Results for sagittal calf thickness showed no significant main effects for time in soleus at SOL_{Th75} (p=0.217), SOL_{Th67} (p=0.141) and a trend in SOL_{Th50} (p=0.063, *d*=0.49). Significant effects for time were observed in gastrocnemius muscle at GAS_{Th75} (p<0.001) and GAS_{Th67} (p=0.004) but not GAS_{Th50} (p=0.468). *Post hoc* analysis revealed that for GAS_{Th75}, T2 and T3 were significantly increased from T1 by 9.7±10.7% (p=0.002) and 18.6±27.7% (p=0.011) respectively. GAS_{Th67} was significantly increased by 20.6±34.7% (p=0.012), T1 to T3.

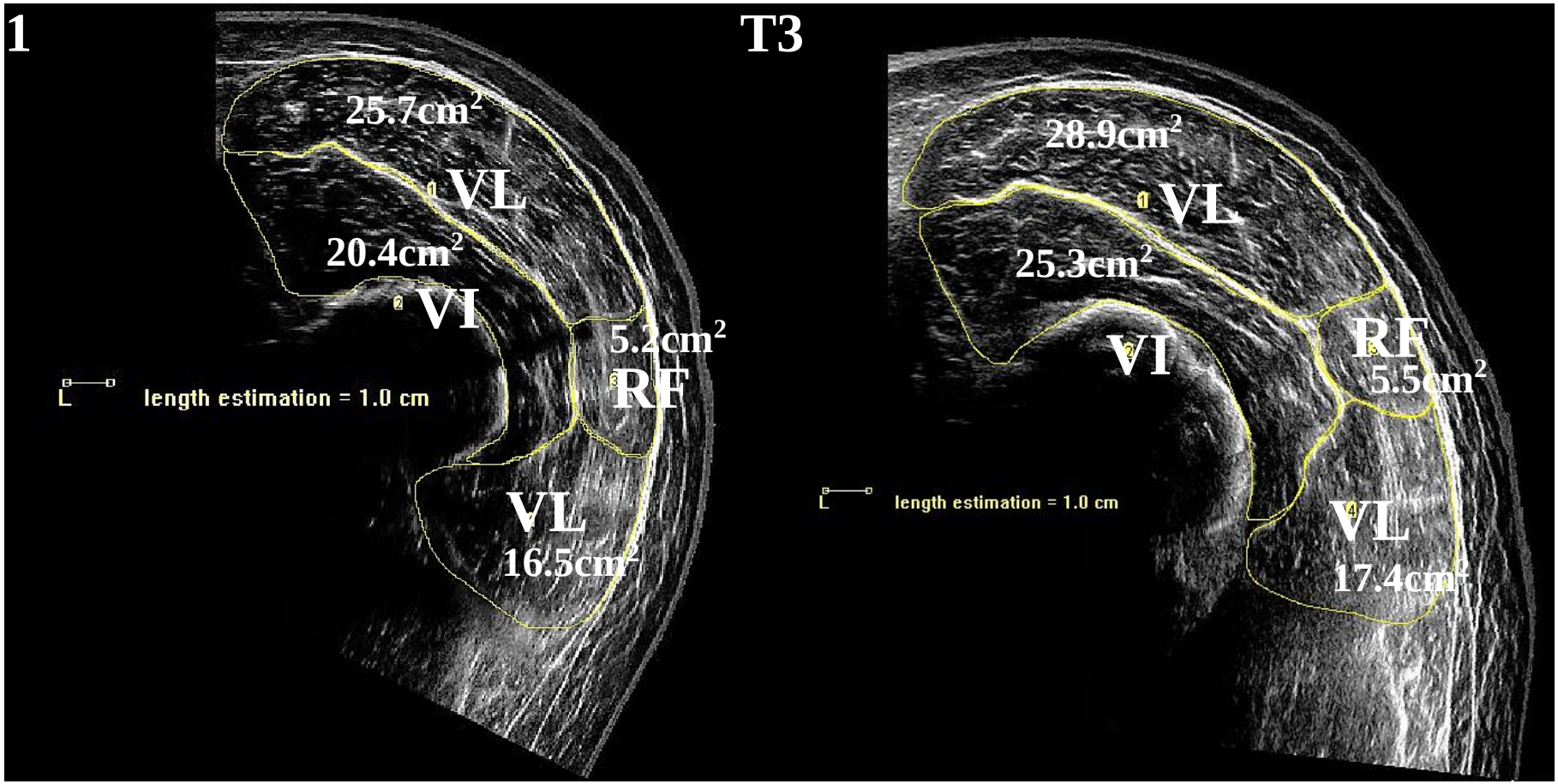


Figure 22 Representative example of quadriceps hypertrophy. Vastus lateralis, VL; vastus intermedialis, VI; rectus femoris, RF; vastus medialis, VM).

Table 14 Quadriceps muscles hypertrophy and morphological changes. Vastus lateralis (VL), vastus intermedialis (VI), rectus femoris (RF), vastus medialis (VM). Ultrasound parameters: CSA slice at 33% distance from centre knee joint to the ASIS ($_{C}$); sagittal thickness at 33% distance from centre knee joint to the ASIS ($_{Th33}$), sagittal thickness at 50% distance from centre knee joint to the ASIS ($_{Th50}$), angle of pennation at 50% distance from centre knee joint to the ASIS ($_{A}$).

	Placebo (n=9)			6 g/d (n =10)		
	T1	T2	T3	T1	T2	T3
CSA						
VL_C (cm^2)	22.4 ± 5.3	24.1 ± 4.9$^{\#}$	24.8 ± 4.7$^{\#\#\#}$	21.1 ± 4.4	21.9 ± 3.6$^{\#}$	22.7 ± 4.1$^{\#\#\#}$
VI_C (cm^2)	23.7 ± 4.1	24.4 ± 3.9	25.3 ± 3.6$^{\#\#\#}$	26.6 ± 3.7	27.3 ± 3.9	27.9 ± 3.8$^{\#\#\#}$
RF_C (cm^2)	4.1 ± 1.4	4.3 ± 0.8	4.6 ± 1.0	4.7 ± 1.2	5.1 ± 1.1	5.2 ± 1.2
VM_C (cm^2)	18.7 ± 4.1	19.6 ± 4.3$^{\#\#\#}$	20.4 ± 4.1$^{\#\#\#}$	18.3 ± 4.6	20.1 ± 4.8$^{\#\#\#}$	20.5 ± 4.7$^{\#\#\#}$
Thickness						
VL_{Th33} (mm)	27.5 ± 3.7	28.5 ± 3.1	28.3 ± 4.0	26.4 ± 4.1	27.1 ± 4.9	27.7 ± 4.8
VI_{Th33} (mm)	17.2 ± 3.4	18.9 ± 2.7	18.4 ± 3.3	19.1 ± 4.0	19.5 ± 4.2	19.8 ± 3.4
VL_{Th50} (mm)	25.8 ± 3.6	26.6 ± 3.5	26.6 ± 4.6	26.0 ± 4.7	27.1 ± 5.5	27.1 ± 4.7
VI_{Th50} (mm)	17.2 ± 3.4	18.9 ± 2.7	18.4 ± 3.3	20.1 ± 4.6	20.6 ± 4.7	20.5 ± 4.6
Angle						
VL_A (degrees)	14.4 ± 2.6	15.6 ± 3.0	16.4 ± 3.7	15.9 ± 3.4	17.5 ± 4.6	16.6 ± 3.2

Data are mean±SD. $^{\#}$ significantly different from T1, irrespective of group ($p<0.05$), $^{\#\#\#}$ ($p<0.001$)

Table 15 Calf muscles hypertrophy. Soleus (SOL), gastrocnemius (GAS). Ultrasound parameters: sagittal thickness at 75% distance from the lateral malleolus to the fibular head ($_{Th75}$), sagittal thickness at 67% distance from the lateral malleolus to the fibular head ($_{Th67}$), sagittal thickness at 50% distance from fibular head to the lateral malleolus ($_{Th50}$).

	Placebo (n=9)			6 g/d (n =10)		
	T1	T2	T3	T1	T2	T3
Soleus						
SOL_{Th75} (mm)	14.0 ± 2.5	13.3 ± 2.6	14.0 ± 2.3	14.1 ± 2.1	13.6 ± 1.5	14.2 ± 1.4
SOL_{Th67} (mm)	14.3 ± 2.3	14.7 ± 2.2	14.9 ± 2.2	14.1 ± 2.2	14.3 ± 2.2	14.9 ± 2.3
SOL_{Th50} (mm)	14.8 ± 2.2	17.4 ± 5.7	16.2 ± 3.0	14.2 ± 3.5	15.5 ± 2.6	16.1 ± 2.2
Gastrocnemius						
GAS_{Th75} (mm)	8.6 ± 3.0	9.4 ± 3.2$^{\#\#}$	10.4 ± 2.4$^{\#}$	9.4 ± 1.6	10.1 ± 1.5$^{\#\#}$	10.1 ± 1.5$^{\#}$
GAS_{Th67} (mm)	8.5 ± 3.0	9.8 ± 3.0	10.6 ± 2.9$^{\#}$	9.4 ± 2.5	9.7 ± 3.0	10.1 ± 2.5$^{\#}$
GAS_{Th50} (mm)	3.8 ± 2.0	4.2 ± 1.8	4.4 ± 1.3	4.5 ± 3.0	3.2 ± 1.0	3.8 ± 0.9

Data are mean±SD. # significantly different from T1, irrespective of group ($p<0.05$), ## ($p<0.01$)

Hormonal relationships with training variables

Testosterone

No relationships were observed between change in total testosterone and change of the sum of quadriceps CSA, at any time point ($p>0.05$). No relationships were found between change in testosterone and change in the isometric strength of the leg extension or calf raise, at any time point ($p>0.05$).

Estradiol

No relationships were observed between change in estradiol and change of the sum of quadriceps CSA, at any time point ($p>0.05$). No relationships were observed between estradiol change and change in the isometric strength of the leg extension, or calf raise at any time point ($p>0.05$). Change scores from T1 to T3 revealed a negative relationship observed between estradiol and soleus iH_{max} $p=0.023$, $R^2=0.269$ (Figure 23)

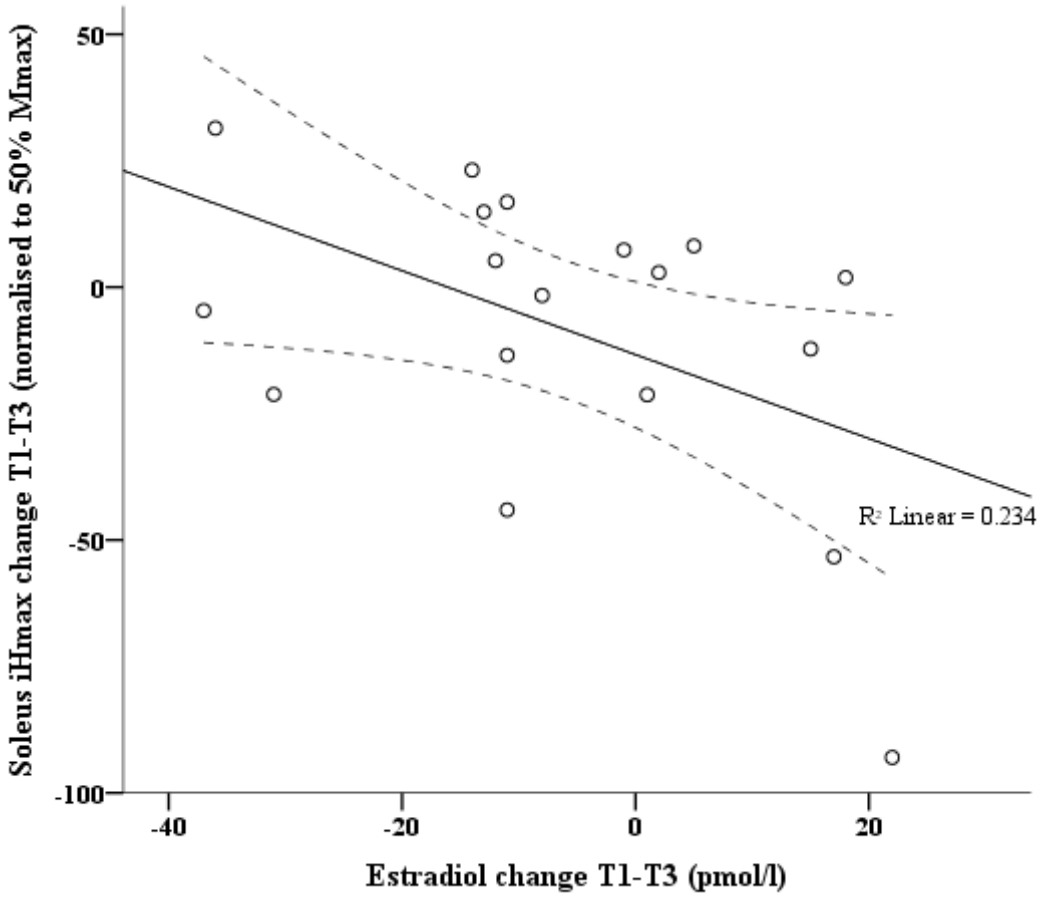


Figure 23 Relationship between estradiol change and change in H_{max} current of the soleus muscle, from T1 to T3. Trend line displays data for both groups (n=18), with a significant association between E_2 and soleus iH_{max} (adjusted R^2=0.186, standardised β coefficient=−0.484, p=0.042). Dashed lines represent 95% CI.

From T2 to T3 a positive relationship was observed between estradiol and both soleus and gastrocnemius H_{slp} variables, p=0.032, R^2=0.257 (Figure 24, soleus); p=0.005, R^2=0.393 (Figure 25, gastrocnemius).

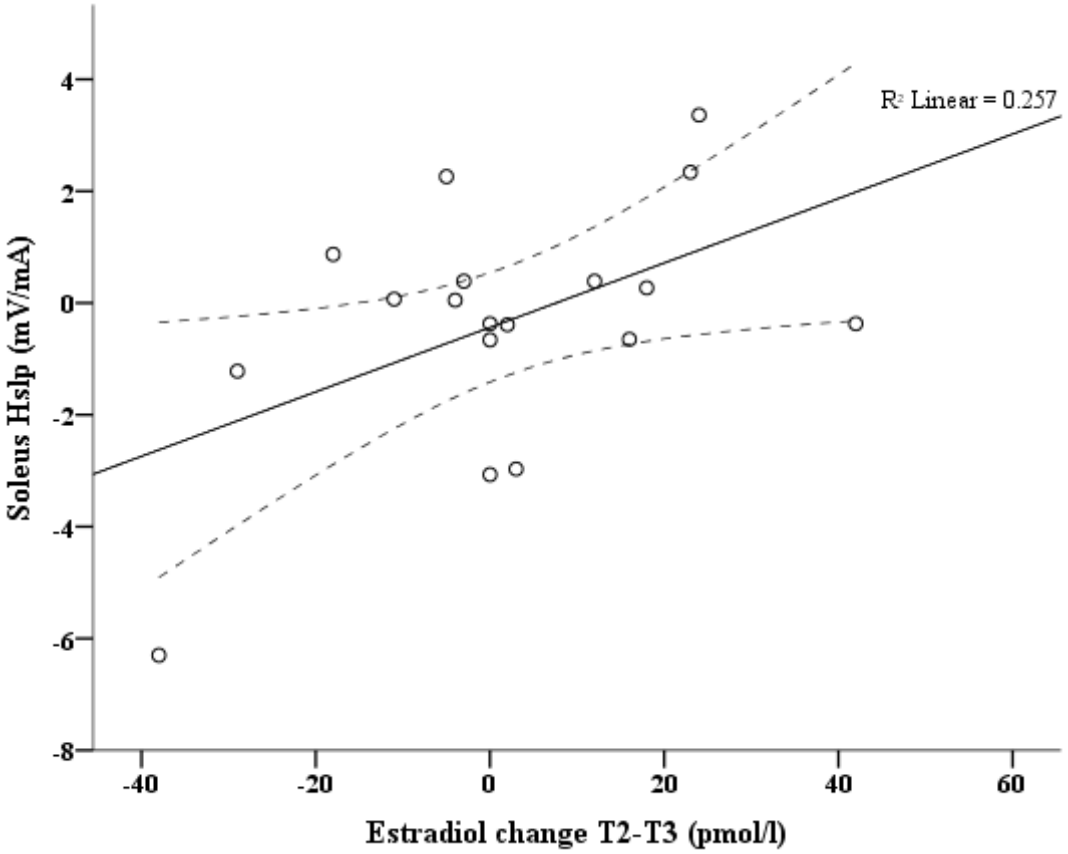


Figure 24 Relationship between change in estradiol and the change in H-reflex slope variable of the soleus muscle, from T2 to T3. Trend line displays data for both groups (n=18), with a significant association between E_2 and soleus H_{slp} (adjusted R^2=0.211, standardised β coefficient=0.507, p=0.032). Dashed lines represent 95% CI.

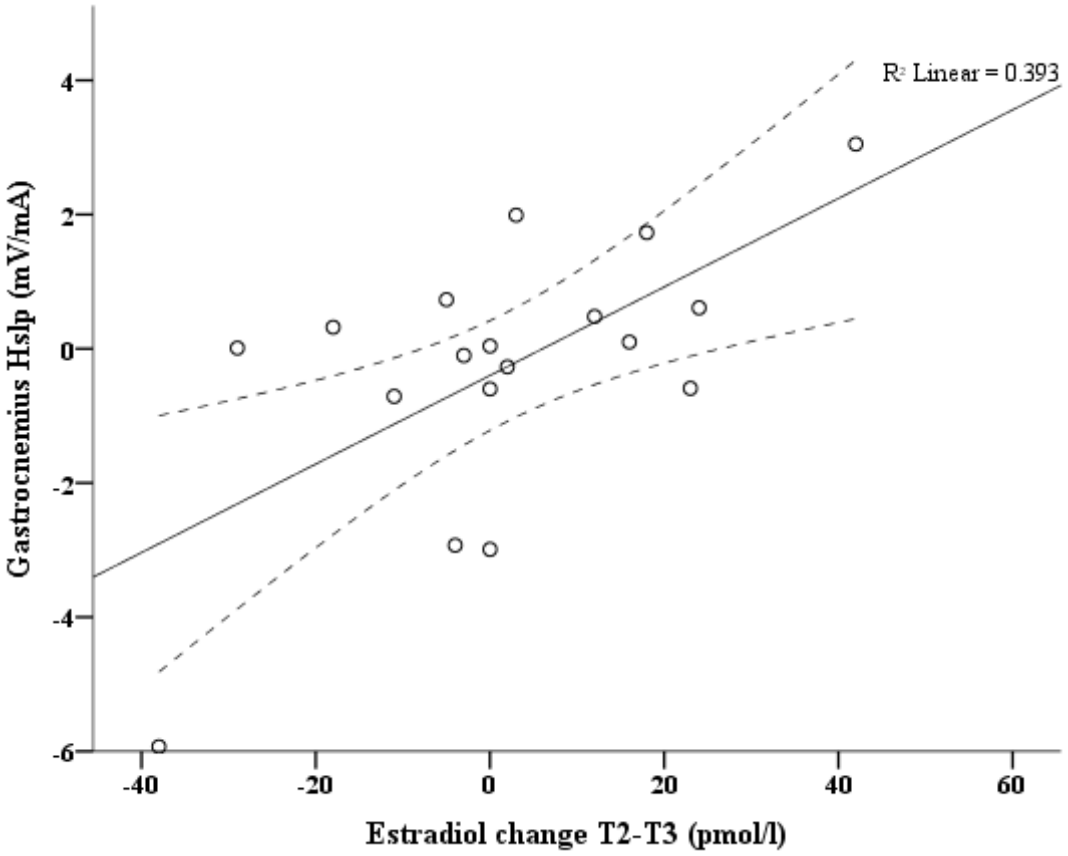


Figure 25 Relationship between estradiol and the H-reflex slope variable of the gastrocnemius muscle. Trend line displays data for both groups (n=18), with a significant association between E_2 and gastrocnemius H_{slp} (adjusted R^2=0.355, standardised β coefficient=0.627, p=0.005). Dashed lines represent 95% CI.

CHAPTER 8 – TRAINING STUDY DISCUSSION

The primary aim of the training study was to investigate the long-term training outcomes, with the supplementation of d-aspartic acid in resistance trained men. The main objectives were to evaluate the effectiveness of six grams of d-aspartic acid on hormonal, strength, hypertrophy and neural change. The principal findings of the present study were, 1) 12 weeks of resistance training resulted in equivalent strength and hypertrophy gains across both the placebo and d-aspartic acid groups, 2) 12 weeks of daily d-aspartic acid supplementation was ineffective at altering total testosterone levels, but reduced levels of estradiol, 3) 12 weeks of daily d-aspartic acid supplementation resulted in an increase in the early rate of force development of the isometric leg extension and 4) the placebo group experienced a reduction in all gastrocnemius h-reflex parameters pertaining to current intensity.

Effects of d-aspartic acid on hormones, strength and hypertrophy

Supplement companies are purporting d-aspartic acid as a viable supplement to be utilised for improving strength and hypertrophy, via a proposed increase in total testosterone. As such the results of the present study refute these claims, and are in accordance with research on d-aspartic acid (3 g/d, 4 weeks) in a resistance trained population (Willoughby & Leutholtz, 2013). Willoughby & Leutholtz observed equivocal gains in strength and hypertrophy in both placebo and d-aspartic acid groups, as well as no change in total testosterone levels or other hormones tested. Recent research has indicated that utilising a daily dose of six grams of d-aspartic acid, decreased levels of total testosterone (Melville et al., 2015), over a two-week

supplementation period (study 1). The lack of change observed in the present study suggests that this reduction of basal testosterone is transitory.

A plausible mechanism for lack of testosterone change in Study 2 of this thesis, could be the upregulation of d-aspartate oxidase production which degrades d-aspartic acid. Previous research in resistance trained men has observed marked increases in d-aspartate oxidase (Willoughby & Leutholtz) with no change in total testosterone levels. Potentially DDO could be acting as a negative feedback mechanism for d-aspartic that regulates levels within the body. Alternatively, negative feedback mechanisms of the hypothalamic–pituitary–gonadal axis could be regulating testosterone synthesis (Figure 4). A decrease in testosterone will cause the hypothalamus to produce more gonadotropin-releasing hormone, stimulating luteinizing hormone production in the pituitary gland, which in turn would travel to the gonads to produce more testosterone (Molina, 2013; Sower, Freamat, & Kavanaugh, 2009). Tying in with this theory could be the effect that estradiol has on feedback mechanisms. Estradiol appears to play a role in the downregulation of the pulse frequency of luteinizing hormone from the anterior pituitary (Hayes, Seminara, Decruz, Boepple, & Crowley Jr, 2000; Rochira et al., 2006). Luteinizing hormone is secreted in a pulsatile manner, that is, it is secreted in short bursts from the hypothalamus rather than as a constant flow. Changes in the pulse frequency of a hormone, therefore, affects the influence that particular hormone imparts on target tissues and negative feedback mechanisms. A decrease in estradiol may cause the pulse frequency of luteinizing hormone to increase (Hayes, Seminara, Decruz, Boepple, & Crowley Jr, 2000; Rochira et al., 2006), which in turn would increase testosterone. Therefore the transitory reduction in total testosterone observed in study 1 (Melville et al., 2015) could have reversed this effect from estradiol or other negative feedback loops (hypothalamus, anterior pituitary gland) over the longer

timeframe of the current study. A potential reason why total testosterone did not continue to increase, is likely because once levels normalised, negative feedback signals to the hypothalamus and anterior pituitary would downregulate production of testosterone (refer to Figure 4), effectively reducing secretion rate to maintenance levels. The combination of these two feedback mechanisms would also likely counteract the effect of the estradiol feedback loop, as estradiol had decreased by the end of the study.

The reduction in estradiol in the current study might be explained by the relative dosage and length of the protocol, as this is the first long-term study with a dosage of six grams of d-aspartic acid, in healthy resistance-trained men. 17β-estradiol is biosynthesized from testosterone in a number of tissues that express the enzyme aromatase. Aromatase is produced from the CYP 19 gene and is part of the cytochrome P450 superfamily (Simpson et al., 2002). In men, approximately 15% of estradiol is synthesised in the testes (Simpson et al., 2002), with the remaining produced at tissues that express aromatase including Leydig cells (testes), adipose tissue, hypothalamus, bone tissue and vascular smooth muscle (Simpson et al., 2002). A potential mechanism of this reduction could have occurred via disruption of the testosterone-estradiol aromatase pathway. D-aspartic acid in relation to estradiol regulation has not been clarified in humans. Furthermore, it is not clear if d-aspartic acid functions differently, depending on the target tissue where the aromatase enzyme is expressed. *In vitro* research on boar testes demonstrate that d-aspartic acid induced a significant increase in aromatase activity (defined as the rate of *in vitro* conversion of testosterone to estradiol) (Lamanna, Assisi, Botte, & Di Fiore, 2007). Direct relationships between d-aspartic acid and estradiol have also been observed in the reproductive cycle of the female lizard (Podarcis s. silica) (Assisi, Botte, D'Aniello, & Di Fiore, 2001).

Moreover, the addition of d-aspartic acid into incubation wells containing lizard ovarian tissue results in an increase in the rate of conversion of testosterone to estradiol. Of note, is that the synthesis of new aromatase is not possible within an *in vitro* model, and while the authors did not ascertain the mechanism of the upregulation of aromatase activity, it was proposed that there were inactive pools of aromatase that was stimulated by the introduced d-aspartic acid (Assisi et al., 2001). Conversely, in the testes of the lizard, a negative correlation is observed between endogenous levels of d-aspartic acid and estradiol levels. Furthermore, injection of d-aspartic acid temporarily reduces estradiol (baseline levels within 24 hours) (Raucci, D'Aniello, & Di Fiore, 2005). These studies suggest that it is possible that the action of d-aspartic acid is dependent on the tissue where it is produced, (or the tissue where it accumulates, in the case of supplementation) which would explain intersex and interspecies differences. In rat's concentrations of estradiol do not change after one or five hours in serum (A. D'Aniello et al., 2000; Santillo et al., 2014). In humans (resistance trained men) estradiol remains unchanged over two (Melville et al., 2015), and four weeks (Willoughby & Leutholtz, 2013) (combined supplementation and resistance training protocols). If the standard function of d-aspartic acid in humans is to upregulate aromatase activity – similar to the changes observed in boar and the female lizard (Assisi et al., 2001; Lamanna et al., 2007) – then it is likely that the higher dose utilised in the present study, had a negative impact on normal functioning of d-aspartic acid on aromatase activity via oversaturation of the tissues with d-aspartic acid. Conversely, if the standard function of d-aspartic acid is to downregulate aromatase activity in humans, which occurs in the male lizard (Raucci et al., 2005), then the accumulation of d-aspartic acid may have caused a gradual reduction in estradiol over time, via reduced aromatase activity. Despite this decrease in estradiol, results of the present

study clearly demonstrate no concurrent differences in hypertrophy or strength gains. This data indicates that the relative effect of this reduction, with respect to gaining muscle or improving strength is inconsequential in comparison to the intrinsic mechanisms driving hypertrophy (A. M. Gonzalez et al., 2016; West, Burd, Staples, et al., 2010) and neural change (Aagaard, 2003; Aagaard et al., 2002b; Van Cutsem et al., 1998). Within the context of neural adaptation, the placebo group experienced increased excitability of the calf H-reflex pathway, which was absent in the d-aspartic acid group. In addition, a positive relationship was observed between estradiol and the recruitment gain of both soleus and gastrocnemius muscles, suggesting the reduction in estradiol might have played a role in the lack of neural change observed in the experimental group.

Effects of d-aspartic acid on the H-reflex pathway

In gastrocnemius, the normalised current intensity for iH_{max} decreased within the placebo group from T2 to T3 (~25%) while there was no change observed in the d-aspartic group. Furthermore, iH_{50} in the placebo group was also was significantly different to d-aspartic acid from T2 to T3. It was hypothesised that d-aspartic acid may improve excitability and neural adaptation via testosterone interaction with androgen receptors within the neurons (Fraley & Ulibarri, 2002; Hammond et al., 2001; Herbst & Bhasin, 2004) or directly via d-aspartic acid's proposed role as a neurotransmitter (A. D'Aniello, 2007; S. D'Aniello et al., 2011; Ota et al., 2012). Contrary to this, results within the gastrocnemius muscle indicated that the placebo group experienced improved excitability of the Ia reflex arc. Improvements of this reflex arc can be represented by an increased amplitude of the H-wave response at a given stimulus, or a reduction of current required to recruit a known H-wave amplitude (for example H_{max}

intensity-). These improvements signify increased excitability at the level of the motoneuron. Previous research in novice individuals has shown that the stimulus intensity required to evoke H-reflex threshold was reduced post training of the dorsiflexors (Dragert & Zehr, 2011) and reduced after plantar flexion strength training (Vila-Cha et al., 2012). Increases have also been observed in H-reflex amplitudes elicited at an equivalent M wave of 5% M_{max} (Lagerquist et al., 2006) and 20% (Holtermann et al., 2007) of M_{max} Conversely some studies have demonstrated no change in various H-reflex variables. Eccentric training of the trapezius muscle in healthy subjects failed to induce any changes in normalised current intensities (Vangsgaard, Taylor, Hansen, & Madeleine, 2014). Eccentric focused training did not change H-reflex elicited at 20% M_{max} during a maximal contraction protocol (Nordlund, 2010) and likewise, maximal strength training at 85-90% 1RM had no effect on the excitability of H-waves recruited at 20% MVC at a current inducing an M-wave 10% of the maximum response. The aforementioned studies suggest that the adaptability of the spinal reflex is quite variable in novices, and when it is observed to change, it tends to improve excitability of the low-threshold motor units. As the results of the current study showed a decrease in the current required to elicit H_{max} and H_{50}, and as motor units are recruited in sequence according to the size principle, regardless of training (Van Cutsem et al., 1998), this suggests that the moderate to high threshold motor units were recruited at a lower stimulus. This is the first study in a resistance trained population, which has observed improvements in spinal responsiveness at the level of the motoneuron similar to previous observations in novice populations (Dragert & Zehr, 2011; Lagerquist et al., 2006; Vila-Cha et al., 2012). With H-reflex methodology it difficult to know where along the spinal pathway this adaptation is occurring. Mechanistically this could be caused by improved excitability of the alpha

motor neuron, improved excitability of the Ia afferent loop, or a decrease in presynaptic inhibition at the level of the interneuron (Zehr, 2002). These improvements of excitability experienced by the placebo group were absent in the d-aspartic acid group.

The lack of change in gastrocnemius h-reflex parameters within the d-aspartic acid group suggests that supplementation is inhibiting adaptation of the excitability of the spinal pathway in the calves. One potential explanation for this could be related to the possible role of d-aspartic acid as a neurotransmitter. Free d-aspartic acid has been demonstrated to have the ability to enter neurons via L-glutamate transporters (Kanai & Hediger, 1992; Koyama et al., 2005). While this could be beneficial in small amounts by providing an additional neurotransmitter to upregulate neural transmission, a large concentration of DAA entering the neuron could be detrimental. If d-aspartic acid is saturating the L-glutamate transporters, then it may be stalling the release and transport of a more potent neurotransmitter, such as glutamate (Engelsen, 1986). While this could be a minor effect, over the course of a training period it may have resulted in the blunted neural adaptation that was observed in the d-aspartic group. Although there was an observed inhibition of adaptation of the spinal pathway in the d-aspartic acid group, both groups equally improved calf isometric and dynamic strength with no change in power within the calf muscles. This suggests that the between-group differences observed in excitability of the spinal pathway to the calf muscles were negligible, with respect to altering strength or power in the calf raise.

Effects of d-aspartic acid on rate of force development

A novel finding of the current study was that early RFD in the leg extension increased in the d-aspartic acid group by ~26.7±26.1% (0-25 ms) and ~30.3±29.7% (0-50 ms), with no change observed in the placebo. These results suggest that the supplementation of six grams of d-aspartic acid improved the participant's ability to rapidly generate force, providing preliminary evidence that suggests that d-aspartic acid could be beneficial for sporting applications which require explosive movement.

Previous research on early RFD change from resistance training conflicts with evidence demonstrating increases in early RFD (Aagaard et al., 2002b; Blazevich, Cannavan, Horne, Coleman, & Aagaard, 2009; de Oliveira, Rizatto, & Denadai, 2013), no change (Häkkinen, Alen, & Komi, 1985) and also decreases (Marshall et al., 2011). Of the aforementioned studies only a few have been conducted in resistance trained populations (Häkkinen, Alen, et al., 1985; Marshall et al., 2011), so the standard response within trained populations has not been clarified. Potential mechanisms that are associated with changes in power include, changes to the intrinsic muscle contractile properties, like twitch RFD (Andersen & Aagaard, 2006), and neural adaptation. Neural adaptation can include: earlier recruitment of type II motor units, as demonstrated by a reduction in recruitment threshold distributions (Van Cutsem et al., 1998); increased observation of 'true' doublet spikes; or increased maximal firing frequency of motor units during voluntary ballistic contractions shown by a reduction of inter-spike intervals (Van Cutsem et al., 1998). Improvements in power have also been observed in traditional periodised training, increases in the mean average EMG (MAV) and the rate of EMG rise (RER), signified an increase in efferent neural drive to the muscle (Aagaard, Simonsen, Andersen, Magnusson, & Dyhre-Poulsen, 2002a).

Furthermore, since they were observed during the early phase of muscle contraction (0-50ms), this suggested changes in motoneuron discharge rate. Authors also reported an increased V-wave response, reflecting an increase in spinal motoneuronal output, which could result in an increase in the firing frequency of the motor units (Aagaard et al., 2002a). The supplementation of d-aspartic acid could also be affecting neural adaptation directly.

D-aspartic acid has been recently suggested to have various properties that are similar to neurotransmitters (A. D'Aniello, 2007). It has been found in high concentrations in various nervous tissues of a marine mollusc and is observed to be released from the synaptic vesicles along with other excitatory amino acids (Spinelli et al., 2006). Additional experiments revealed that injection of d-aspartic acid into these animals increased cAMP content, analysed within the cerebral ganglia. Live cultured neurons, extracted from the cerebral ganglia, also resulted in an increase in cAMP content (Spinelli et al., 2006). The importance of this finding, is further evidence of d-aspartic acid's role as a neurotransmitter, as various cellular responses to neurotransmitters rely on the cAMP molecule via the second messenger pathway (Spinelli et al., 2006). Research has also suggested that d-aspartic acid may be able to use L-glutamate transporters (Koyama et al., 2005), which could be a potential mechanism for how free d-aspartic acid in the blood, could enter the neuroglia and the neurons (Spinelli et al., 2006). It is not clear from the research if d-aspartic acid's role within the brain would differ to its potential role within the periphery. It is hard to speculate the specific mechanisms which are attributing to the change in leg extension RFD of the current study, due to the lack of neural testing within the quadriceps.

Hypothetically as d-aspartic acid appears to have various properties akin to neurotransmitters, supplementation may have increased the availability of d-aspartic

acid within the brain or neurons, which improved cortical or spinal excitability and resulted in an improvement in RFD. As the change in RFD was exclusive to the quadriceps and not the calf muscles, it is likely that this adaptation occurred as a combination of specific programming and interaction with d-aspartic acid supplementation. The lack of change in RFD within the calf muscles may be explained by the amount of volume prescribed, which was lower than the quadriceps prescription. Training volume for the calf muscles encompassed 36 reps in the 10RM – 8RM range and 6 reps at 6RM during the first 6-week block. In weeks 7 – 12, there was 26 reps at 10RM – 8RM and 12 reps at 6RM. Additional volume for the calf included 30 reps at approximately 20RM – 30RM (sets to failure). Conversely, quadriceps volume during weeks 1 – 6 was comprised of 114 reps per week in the 10RM – 8RM range and 28 reps within the 6RM – 8RM. Volume during weeks 7 – 12 included 82 reps at the 10RM – 8RM range and 48 reps at 6RM. Thus for the quadriceps, the initial six weeks utilised high volume in the moderate intensity range, with less volume at the high-intensity range. The second periodisation block, traded volume in the moderate intensity range, for increased volume at the high-intensity range. Similar periodisation changes have previously resulted in improvements in early and peak RFD in novices (Aagaard et al., 2002a). Aagaard and colleagues implemented a training protocol which required 3RM – 10RM training for 10 weeks and then implemented a 4RM – 6RM heavy loading cycle for the final month. In novices, it is possible that a periodisation change to greater training volume at higher intensities is driving RFD improvements from resistance training. The placebo results of the current study revealed that the training alone did not elicit an RFD change in resistance trained men, despite similar volume and intensity changes as the study conducted by Aagaard and colleagues. Even though power improvements have been

observed with resistance training in novices (Aagaard et al., 2002a), specific power training may be required to see continual RFD improvements in a resistance trained population, as research has shown that in these populations resistance training either, does not change RFD, or can even decrease RFD (Häkkinen, Alen, et al., 1985; Marshall et al., 2011). Despite this, d-aspartic acid supplementation resulted in increased early RFD within the quadriceps, albeit not until post testing. It is reasonable to believe that the periodisation change at the midpoint of this study, to more volume at the higher intensity range (4RM – 6RM), in combination with supplementation, triggered the positive adaptation in power. As this is the first study to observe d-aspartic acid mediated improvements in RFD, further research is required to investigate these preliminary results and delineate the mechanisms that are causing the enhancement of early RFD within the quadriceps.

Effects of d-aspartic acid on supraspinal drive

The present study showed that V/M_{max} ratio in the calf muscles was not significantly different between groups, neither did V/M_{max} improve over the course of the training study. These results suggest that neither d-aspartic acid nor the stimulus of resistance training was able to change supraspinal drive to the calf muscles in this population. Research in novice populations has demonstrated increases in V/M_{max} ratio with resistance training (Aagaard et al., 2002b; Del Balso & Cafarelli, 2007; Nordlund, 2010; Vila-Cha et al., 2012), additionally when RT individuals are compared with novices, significantly larger V-wave responses are observed in the trained individuals (Sale et al., 1983). Data on endurance training has failed to show any improvements in V-wave measures, suggesting that the enhancement of neural drive is an adaptation exclusive to resistance training (Vila-Cha et al., 2012). The results from the present

study exhibited high variability between subject's V-waves, which might suggest an issue with the sensitivity of the method that was used in this study. Alternatively, given the nature that calves are not a high priority muscle to train, it could suggest that data obtained from the participants represents a population with a mixture of trained and untrained calves. Regardless, the variability in the data suggests that the study could have been underpowered to observe a significant change in V-waves. Furthermore, alterations in supraspinal drive cannot be entirely eliminated with the methodology used in the current study. This is due to the fact that changes in V-waves are an indirect measure of supraspinal drive. V-waves can be affected by spinal mechanisms, such as alterations within the Ia afferent pathway or changes at the level of the motoneuron. With the absence of a direct measure of supraspinal drive, such as transcranial magnetic stimulation, it is possible that a change may have occurred in neural drive that was not detected with the current methodology. This is the first study to investigate changes in V-waves, in a resistance trained population over a long-term training study. The lack of change within the placebo group could suggest that improvements in supraspinal drive do not influence strength changes in resistance trained populations. Alternatively, it is plausible that the V-wave methodology of the current study was not sensitive enough to observe a change in a resistance trained population.

Importance of sex hormones in trained populations

A secondary objective of this thesis was to investigate if any relationships between hormones and improvements in strength, hypertrophy or neural adaptation, exist within a resistance trained population.

Total testosterone

It was hypothesised that testosterone would have a positive relationship with hypertrophy and isometric strength. No relationships were observed between resting total testosterone change and change in the sum of quadriceps CSA, nor with a change in leg extension MVC or change in calf raise MVC. Previous research has found positive relationships between maximal isometric force and the hormonal milieu in both resistance trained (Häkkinen, Pakarinen, et al., 1985) and novices (Ahtiainen et al., 2003). One study has explored the relationship between testosterone and hypertrophy, which investigated the effects of various dosages of testosterone enanthate (Sinha-Hikim et al., 2002). Change in type I and II muscle fibre area were observed to be positively related to the change in testosterone, irrespective of any training stimulus. The difference between this study and the present study, however, is the relative shift in testosterone levels. In the present study, no significant changes were observed in testosterone levels over the course of the study, despite observed changes in hypertrophy. Conversely, testosterone enanthate doubled (300mg) and tripled (600mg) serum testosterone, bringing levels well above clinical ranges. With this amount of testosterone in the blood the likelihood of interaction with androgen receptors is markedly increased, resulting in the relationship with fibre area hypertrophy that was observed (Sinha-Hikim et al., 2002). The results of the present

study suggest that in a resistance trained population, resting testosterone levels do not significantly change over 12 weeks of periodised training. There were also no drastic changes to volume or intensity which could have triggered an overtraining effect that has been observed previously, nor did d-aspartic acid appear to have any long-term effect on testosterone. Resistance trained populations tend to exhibit higher levels of testosterone (Häkkinen et al., 1988a; Häkkinen, Pakarinen, et al., 1985; McCall et al., 1999; Reaburn et al., 1997; Staron et al., 1994; Willoughby & Leutholtz, 2013), which appears to be a chronic adaptation, as a result of a year or more continuous training. After this adaptation is realised, provided there is no obvious change in volume or intensity (Häkkinen et al., 1987), resting testosterone levels may be relatively stable in resistance trained populations. These results suggest that the importance of basal testosterone change, within clinical ranges, are negligible in resistance trained populations, with respect to strength or hypertrophy improvements. The data presented in this study is further evidence that serum levels of hormones play a more of a permissive role in hypertrophy. These results also highlight that it is likely that intrinsic factors such as activation of various signalling pathways, phosphorylation of intramuscular signalling proteins, regulation of messenger RNA (translation initiation), increases in AR content and satellite cell activity that play a larger role in the hypertrophic response to resistance training (A. M. Gonzalez et al., 2016; West, Burd, Staples, et al., 2010).

Estradiol

A positive relationship was observed between the change in estradiol and the change in the H-reflex slope parameters of soleus and gastrocnemius from, T2 to T3. The slope of the ascending limb of the H-reflex curve represents a reduction in the degree of

threshold differences within the motoneuron pool, or an improvement in the ease of recruitment gradation (Kernell & Hultborn, 1990; Vila-Cha et al., 2012). Estradiol is understood to play a role in the functioning of the central nervous system. *In vitro* hippocampal slices from the rat brain have demonstrated that estradiol enhances synaptic transmission and the degree of long-term potentiation (Foy, 2011). In women, stages of the menstrual cycle with high estradiol levels (late follicular to mid luteal phase), were associated with less inhibition and more facilitation activity when probed with a weak magnetic stimulus (TMS). Conversely, there was a tendency for higher inhibition, when circulation estradiol was low during the early follicular phase (Smith et al., 1999). Whilst there is less data on the effects of estradiol in men, the regression results suggest that reduced levels of estradiol may decrease the excitability of the spinal Ia afferent pathway. A negative relationship was also observed between the change in estradiol and the change in the current for H_{max} within the soleus muscle, from T1 to T3. This relationship is indicating that a reduction in estradiol tended to increase the stimulus required to elicit H_{max}, suggesting that those individuals with lower estradiol at post-testing negatively impacted excitability, at the level of the motoneuron.

Limitations

This study has a few limitations pertaining to methodological design. One potential issue with this research is the reliance on participants taking their supplement dosage each day. Participants were given their allocated supply for six weeks at a time, to help keep track of adherence. With this design, it is possible that some participants might have forgotten to take their daily dose on occasion, despite this being controlled for, via weekly checks and prompts, there can be issues with compliance when participants

are instructed to take supplements on their own time. In hindsight, an ideal system would have participants consuming their pills in front of the primary investigator during all face-to-face interactions, with an online/mobile check-in program to account for all the other days. Another limitation of this study is the lack of neural testing for the quadriceps muscles. Quadriceps data on H-reflex and V-wave changes would provide better insight, as to the mechanism behind the improvement in quadriceps RFD experienced in the DAA group. Neural adaptation measures were piloted in the quadriceps, however, unfortunately, the regular appearance of both H-waves and V-waves could not be replicated. After multiple attempts at different protocols, it was decided in the interest of time management, to omit the neural measures in the quadriceps. This design choice ties in with another limitation in relation to the omission of a clinical marker of dynamic strength. Ideally, a 1RM protocol for the squat could have been included. With the original study design that included quadriceps neural testing, isometric leg extension was chosen to mirror this methodology as 1RM testing of the squat closely represents gross strength across multiple joints, which was believed would not be an ideal comparison with the neural markers. Another reason 1RM testing was omitted due was due to the time constraints of the participants. An extra day of 1RM testing would have been required, so as not to interfere with neural methods and vice versa. Furthermore, we believed that the superior control that isometric testing provides would result in a better indicator of strength change over a 1RM protocol, especially for calf strength which could be more accurately compared to the neural change. In relation to this issue is the choice of 10-RM calf raise and 10-RM leg extension to indicate strength changes. It could be argued that the utilisation of log diary to indicate changes in repetition maximum strength has some shortfalls. However, it is believed that this accurately depicts strength changes

throughout the study intervention as participants were encouraged to undertake progressive loading. Furthermore, we believe despite leg extension and calf raise - while traditionally utilised as an accessory exercise - are an ideal match to our testing protocols of neural and isometric testing, over and above a more traditional testing measure such as the squat (which crosses multiple joints). Another limitation of this research is the reliance on V-waves to measure the change in supraspinal drive. The inclusion of a direct measure of supraspinal drive, such as transcranial magnetic stimulation may have provided clearer insight into potential cortical changes. With respect to hypertrophy, a stronger and more reliable measure for muscular change would have been to use magnetic resonance imagery (MRI). The ease of use and access of ultrasound equipment outweighed the accessibility, cost and organisation required to utilise MRI technology for hypertrophy testing. An additional limitation of this study is the likelihood of being underpowered for some measures, with the potential for type II errors. A number of measures presented trends, with moderate to small effect sizes, suggesting that significant results may have been observed if more participants were recruited.

Conclusion

The results of the present study demonstrate that the supplementation of d-aspartic acid daily for 12 weeks, with resistance training, has no additive benefit on strength, or hypertrophy in the calf or quadriceps muscles over just resistance training alone. D-aspartic acid did not have any long-term positive or negative effects on basal testosterone levels, which could be explained by negative-feedback mechanisms of the body attempting to keep the hormonal state of the body in homoeostasis. Supplementation of d-aspartic acid caused a decrease in resting estradiol levels, which

theoretically could be as a result of disruption of the testosterone-estradiol aromatase pathway. This reduction in estradiol did not appear to influence strength or hypertrophy negatively. However, results from regression analysis suggest that reductions in estradiol could be negatively impacting mechanisms of spinal excitability. The present study demonstrated that supplementation of d-aspartic acid preferentially improved explosive power, in the early phase of the rate of force development. This adaptation was only observed within the quadriceps muscle, which might be explained by a difference in prescribed volume. Moreover, contradictory results were observed in spinal excitability of the calf muscles, whereby preferential adaptation of the H-reflex pathway occurred in the placebo group. If d-aspartic acid is able to improve early rate of force development, then it could be beneficial to sports which require explosive power development. However, more research is needed to confirm these findings and explain the mechanism of d-aspartic acid mediated improvement in power.

CHAPTER 9 – THESIS CONCLUSION

Summary

The aim of this thesis was to undertake a systematic investigation of the effectiveness of the proposed testosterone boosting supplement, d-aspartic acid, in resistance trained men. Chapters 3-5 presented the initial study of this thesis, whereby after a familiarisation period, three doses of d-aspartic acid (0g, 3g and 6g), were given daily to resistance trained men for 14 days to observe changes in basal hormones. This study identified that three grams of d-aspartic acid did not significantly affect hormonal levels within this population. The six-gram dose reduced total testosterone levels by 12.5% and free testosterone by 15.3%. Study 2, was presented in chapters 6-8 and explored the six-gram dosage over a periodised 12-week resistance training program. The experimental group experienced a reduction in estradiol, by the end of the 12 weeks, which is believed to be as a result of disruption of the testosterone-estradiol aromatase pathway. D-aspartic acid also resulted in an increase in early RFD in leg extension, which is likely as a consequence of a combination of the programming prescribed in the second six-week block, and d-aspartic acids proposed role as a neurotransmitter. The placebo group exclusively experienced an improvement of spinal excitability, of the moderate to high threshold motor units within the gastrocnemius muscle. Isometric strength and hypertrophy of the calf and quadriceps muscles increased in both groups.

Originality of work

Study 1 of this thesis was the first study to explore multiple dosages of d-aspartic acid in humans (Melville et al., 2015). Previous research in resistance trained men had failed to show any improvement in testosterone levels with short-term d-aspartic acid supplementation. It was hypothesised that a larger dosage might be required to increase levels within a resistance trained population. A novel finding of study 1, was that the six-gram d-aspartic acid group actually decreased testosterone levels, over a two-week supplementation period. Study 2 is the first long-term training study to explore d-aspartic acid supplementation in resistance-trained men. Additionally, study 2 is the first study to explore spinal plasticity, as measured by H-reflexes, within a resistance trained population. Original findings of study 2 include the reduction in estradiol in the d-aspartic acid group; increase in early rate of force development of the quadriceps (d-aspartic acid group); and blunting of spinal plasticity to the calves, which was only observed in the placebo group. Furthermore, this is one of the first studies to find no relationship between change in testosterone levels and change in hypertrophy or strength.

Practical Implications

For the sole purpose of strength or hypertrophy gains, the results of this thesis suggest no added benefit from taking d-aspartic acid while training if the individual is resistance trained. Thus recommendations are to avoid this supplement if the goal is to train for pure strength or hypertrophy, due to its potential to negatively disrupt hormonal homoeostasis, and no added benefit while training for these goals. Within the context of power development, preliminary data suggests that supplementation of

d-aspartic acid (6 grams per day) improves early rate of force development, within the quadriceps muscles, in resistance trained men. It is possible that supplementation could be applicable to athletes seeking improvement in quadriceps power. In order to take advantage of this potential improvement in power, a periodised program should be implemented, with similar volume and intensity distributions. Furthermore, it should be expected that this power improvement, might be exclusive to the quadriceps until further research is conducted. Since a specific power program was not implemented in this study, the results of this research cannot be extended to this type of programming.

Future directions

Future research should aim to further elucidate a potential mechanism for the improvement in early RFD, within the quadriceps muscle following, d-aspartic acid supplementation and resistance training. Ideally, this research would explore neural adaptation within the quadriceps muscle, and utilise a protocol that could also delineate if adaptation is occurring from presynaptic or postsynaptic inhibition mechanisms. Additionally, a combination of resistance and power training could be explored to provide the greatest stimulus, to observe a different between d-aspartic acid and placebo. The effects of d-aspartic acid on the rate of force development could also be explored within the context of an acute setting. If d-aspartic acid is able to improve early rate of force development from a single dose, or from a loading strategy, there could be a useful application to temporary explosive power improvement, without the added issues of hormonal disruption. Additionally, power adaptations could be explored in other muscles, to delineate if this adaptation is exclusive to the quadriceps.

Conclusion

The results of this thesis support and expand upon the current literature on the supplementation of d-aspartic acid in resistance trained men. D-aspartic acid appears to be negatively disrupting hormonal homoeostasis, which temporarily manifests as a reduction in testosterone. Long term, the decrease in testosterone seems to be corrected by the body, at the expense of a reduction in estradiol. Supplementation has no beneficial effect on strength and hypertrophy. Conversely, d-aspartic acid appears to have some interesting, conflicting effects on neural adaptation and rate of force development; that warrant further investigation to determine if this supplement could still be useful within the correct context.

REFERENCES

Aagaard, P. (2003). Training-induced changes in neural function. *Exercise and Sport Sciences Reviews, 31*(2), 61-67.

Aagaard, P., Simonsen, E. B., Andersen, J. L., Magnusson, P., & Dyhre-Poulsen, P. (2002a). Increased rate of force development and neural drive of human skeletal muscle following resistance training. *Journal of Applied Physiology, 93*(4), 1318-1326.

Aagaard, P., Simonsen, E. B., Andersen, J. L., Magnusson, P., & Dyhre-Poulsen, P. (2002b). Neural adaptation to resistance training: Changes in evoked V-wave and H-reflex responses. *Journal of Applied Physiology, 92*(6), 2309-2318.

Adkins, D. L., Boychuk, J., Remple, M. S., & Kleim, J. A. (2006). Motor training induces experience-specific patterns of plasticity across motor cortex and spinal cord. *Journal of Applied Physiology, 101*(6), 1776-1782.

Ahtiainen, J. P., Hulmi, J. J., Kraemer, W. J., Lehti, M., Nyman, K., Selänne, H., . . . Häkkinen, K. (2011). Heavy resistance exercise training and skeletal muscle androgen receptor expression in younger and older men. *Steroids, 76*(1-2), 183-192.

Ahtiainen, J. P., Pakarinen, A., Alen, M., Kraemer, W. J., & Häkkinen, K. (2003). Muscle hypertrophy, hormonal adaptations and strength development during strength training in strength-trained and untrained men. *European Journal of Applied Physiology, 89*(6), 555-563.

Akima, H., Takahashi, H., Kuno, S. Y., Masuda, K., Masuda, T., Shimojo, H., . . . Katsuta, S. (1999). Early phase adaptations of muscle use and strength to isokinetic training. *Medicine and Science in Sports and Exercise, 31*(4), 588-594.

Alen, M., Pakarinen, A., Häkkinen, K., & Komi, P. V. (1988). Responses of serum androgenic-anabolic and catabolic hormones to prolonged strength training. *International Journal of Sports Medicine, 9*(3), 229-233.

Alessi, D. R., & Cohen, P. (1998). Mechanism of activation and function of protein kinase B. *Current Opinion in Genetics and Development, 8*(1), 55-62.

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R. J., Reese, C. B., & Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Current Biology, 7*(4), 261-269.

Allen, D. L., Roy, R. R., & Reggie Edgerton, V. (1999). Myonuclear domains in muscle adaptation and disease. *Muscle and Nerve, 22*(10), 1350-1360.

Andersen, L. L., & Aagaard, P. (2006). Influence of maximal muscle strength and intrinsic muscle contractile properties on contractile rate of force development. *European Journal of Applied Physiology, 96*(1), 46-52.

Arias, P., Carbone, S., Szwarcfarb, B., Feleder, C., Rodríguez, M., Scacchi, P., & Moguilevsky, J. A. (1996). Effects of aging on N-methyl-D-aspartate (NMDA)-induced GnRH and LH release in female rats. *Brain Research, 740*(1-2), 234-238.

Arslan, M., Pohl, C. R., & Plant, T. M. (1988). DL-2-amino-5-phosphonopentanoic acid, a specific N-methyl-D-aspartic acid receptor antagonist, suppresses pulsatile LH release in the rat. *Neuroendocrinology, 47*(5), 465-468.

Assisi, L., Botte, V., D'Aniello, A., & Di Fiore, M. M. (2001). Enhancement of aromatase activity by D-aspartic acid in the ovary of the lizard Podarcis s. sicula. *Reproduction, 121*(5), 803-808.

Bamman, M. M., Shipp, J. R., Jiang, J., Gower, B. A., Hunter, G. R., Goodman, A., . . . Urban, R. J. (2001). Mechanical load increases muscle IGF-I and androgen receptor mRNA concentrations in humans. *American Journal of Physiology - Endocrinology and Metabolism, 280*(3 43-3).

Barr, K., & Esser, K. (1999). Phosphorylation of p70S6k correlates with increased skeletal muscle mass following resistance exercise. *Am J Physiol, 276*.

Basualto-Alarcón, C., Jorquera, G., Altamirano, F., Jaimovich, E., & Estrada, M. (2013). Testosterone signals through mTOR and androgen receptor to induce muscle hypertrophy. *Medicine and Science in Sports and Exercise, 45*(9), 1712-1720.

Bellamy, L. M., Joanisse, S., Grubb, A., Mitchell, C. J., McKay, B. R., Phillips, S. M., . . . Parise, G. (2014). The acute satellite cell response and skeletal muscle hypertrophy following resistance training. *PLoS ONE, 9*(10).

Berg, H. E., Tedner, B., & Tesch, P. A. (1993). Changes in lower limb muscle cross-sectional area and tissue fluid volume after transition from standing to supine. *Acta Physiologica, 148*(4), 379-385.

Bhasin, S., Cunningham, G. R., Hayes, F. J., Matsumoto, A. M., Snyder, P. J., Swerdloff, R. S., & Montori, V. M. (2010). Testosterone therapy in men with androgen deficiency syndromes: An endocrine society clinical practice guideline. *Journal of Clinical Endocrinology and Metabolism, 95*(6), 2536-2559.

Bhasin, S., Storer, T. W., Berman, N., Callegari, C., Clevenger, B., Phillips, J., . . . Casaburi, R. (1996). The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *New England Journal of Medicine, 335*(1), 1-7.

Bhasin, S., Zhang, A., Coviello, A., Jasuja, R., Ulloor, J., Singh, R., . . . Vasan, R. S. (2008). The impact of assay quality and reference ranges on clinical decision making in the diagnosis of androgen disorders. *Steroids, 73*(13), 1311-1317.

Bischoff, R. (1997). Chemotaxis of skeletal muscle satellite cells. *Developmental Dynamics, 208*(4), 505-515.

Blazevich, A. J., Cannavan, D., Horne, S., Coleman, D. R., & Aagaard, P. (2009). Changes in muscle force-length properties affect the early rise of force *in vivo*. *Muscle and Nerve, 39*(4), 512-520.

Bloomer, R. J., Gunnels, T. A., Moran, R. G., & Schriefer, J. M. (2015). Influence of a d-aspartic acid/sodium nitrate/vitamin d3 dietary supplement on physiological parameters in middle-aged men: A pilot study. *Open Nutraceuticals Journal, 8*(1), 43-48.

Bonifazi, M., Ginanneschi, F., Della Volpe, R., & Rossi, A. (2004). Effects of gonadal steroids on the input-output relationship of the corticospinal pathway in humans. *Brain Research, 1011*(2), 187-194.

Borst, S. E., & Mulligan, T. (2007). Testosterone replacement therapy for older men. *Clinical interventions in aging, 2*(4), 561-566.

Borst, S. E., Vincent, K. R., Lowenthal, D. T., & Braith, R. W. (2002). Effects of resistance training on insulin-like growth factor and its binding proteins in men and women aged 60 to 85. *Journal of the American Geriatrics Society, 50*(5), 884-888.

Bourguignon, J. P., Gerard, A., & Franchimont, P. (1989). Direct activation of gonadotropin-releasing hormone secretion through different receptors to neuroexcitatory amino acids. *Neuroendocrinology, 49*(4), 402-408.

Brook, M. S., Wilkinson, D. J., Mitchell, W. K., Lund, J. N., Szewczyk, N. J., Greenhaff, P. L., . . . Atherton, P. J. (2015). Skeletal muscle hypertrophy adaptations predominate in the early stages of resistance exercise training, matching deuterium oxide-derived measures of muscle protein synthesis and mechanistic target of rapamycin complex 1 signaling. *FASEB Journal, 29*(11), 4485-4496.

Bruce, S. A., Phillips, S. K., & Woledge, R. C. (1997). Interpreting the relation between force and cross-sectional area in human muscle. *Medicine and Science in Sports and Exercise, 29*(5), 677-683.

Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H., & Sabatini, D. M. (1998). RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proceedings of the National Academy of Sciences of the United States of America, 95*(4), 1432-1437.

Chang, W. J., Barb, C. R., Kraeling, R. R., Rampacek, G. B., & Asanovich, K. M. (1993). N-methyl-D,L-aspartate modulation of pituitary hormone secretion in the pig: Role of opioid peptides. *Domestic Animal Endocrinology, 10*(4), 305-313.

Chen, J., & Fang, Y. (2002). A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. *Biochemical Pharmacology, 64*(7), 1071-1077.

Chin, K. Y., Soelaiman, I. N., Naina Mohamed, I., Shahar, S., Teng, N. I. M. F., Suhana Mohd Ramli, E., . . . Ngah, W. Z. W. (2012). Testosterone is associated with age-related changes in bone health status, muscle strength and body composition in men. *Aging Male, 15*(4), 240-245.

Collins, C. A., Olsen, I., Zammit, P. S., Heslop, L., Petrie, A., Partridge, T. A., & Morgan, J. E. (2005). Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell, 122*(2), 289-301.

Craig, B. W., Brown, R., & Everhart, J. (1989). Effects of progressive resistance training on growth hormone and testosterone levels in young and elderly subjects. *Mechanisms of Ageing and Development, 49*(2), 159-169.

Cumming, D. C., Wall, S. R., Galbraith, M. A., & Belcastro, A. N. (1987). Reproductive hormone responses to resistance exercise. *Medicine and Science in Sports and Exercise, 19*(3), 234-238.

D'Aniello, A. (2007). d-Aspartic acid: An endogenous amino acid with an important neuroendocrine role. *Brain Research Reviews, 53*(2), 215-234.

D'Aniello, A., Di Cosmo, A., Di Cristo, C., Annunziato, L., Petrucelli, L., & Fisher, G. (1996). Involvement of D-aspartic acid in the synthesis of testosterone in rat testes. *Life Sciences, 59*(2), 97-104.

D'Aniello, A., Di Fiore, M. M., Fisher, G. H., Milone, A., Seleni, A., D'Aniello, S., . . . Ingrosso, D. (2000). Occurrence of D-aspartic acid and N-methyl-D-aspartic acid in rat neuroendocrine tissues and their role in the modulation of luteinizing hormone and growth hormone release. *FASEB Journal, 14*(5), 699-714.

D'Aniello, G., Tolino, A., D'Aniello, A., Errico, F., Fisher, G. H., & Di Fiore, M. M. (2000). The role of D-aspartic acid and N-methyl-D-aspartic acid in the regulation of prolactin release. *Endocrinology, 141*(10), 3862-3870.

D'Aniello, S., Somorjai, I., Garcia-Fernàndez, J., Topo, E., & D'Aniello, A. (2011). D-Aspartic acid is a novel endogenous neurotransmitter. *FASEB Journal, 25*(3), 1014-1027.

D'Aniello, G., Ronsini, S., Notari, T., Grieco, N., Infante, V., D'Angel, N., . . . D'Aniello, A. (2012). d-Aspartate, a key element for the improvement of sperm quality. *Advances in Sexual Medicine, 2*(4), 45-53.

Damas, F., Phillips, S. M., Libardi, C. A., Vechin, F. C., Lixandrão, M. E., Jannig, P. R., . . . Ugrinowitsch, C. (2016). Resistance training-induced changes in integrated myofibrillar protein synthesis are related to hypertrophy only after attenuation of muscle damage. *Journal of Physiology, 594*(18), 5209-5222.

de Oliveira, F. B. D., Rizatto, G. F., & Denadai, B. S. (2013). Are early and late rate of force development differently influenced by fast-velocity resistance training? *Clinical Physiology and Functional Imaging, 33*(4), 282-287.

Del Balso, C., & Cafarelli, E. (2007). Adaptations in the activation of human skeletal muscle induced by short-term isometric resistance training. *Journal of Applied Physiology, 103*(1), 402-411.

Deldicque, L., Atherton, P., Patel, R., Theisen, D., Nielens, H., Rennie, M. J., & Francaux, M. (2008). Decrease in Akt/PKB signalling in human skeletal muscle by resistance exercise. *European Journal of Applied Physiology, 104*(1), 57-65.

Downing, J. A., Joss, J., & Scaramuzzi, R. J. (1996). The effects of N-methyl-D,L-aspartic acid and aspartic acid on the plasma concentration of gonadotropins. GH and prolactin in the ewe. *J Endocrinol, 149*(1), 65-72.

Dragert, K., & Zehr, E. P. (2011). Bilateral neuromuscular plasticity from unilateral training of the ankle dorsiflexors. *Experimental Brain Research, 208*(2), 217-227.

Duclay, J., Martin, A., Robbe, A., & Pousson, M. (2008). Spinal reflex plasticity during maximal dynamic contractions after eccentric training. *Medicine and Science in Sports and Exercise, 40*(4), 722-734.

Dumont, N. A., Bentzinger, C. F., Sincennes, M. C., & Rudnicki, M. A. (2015). Satellite cells and skeletal muscle regeneration. *Comprehensive Physiology, 5*(3), 1027-1059.

Dunlop, D. S., Neidle, A., & McHale, D. (1986). The presence of free D-aspartic acid in rodents and man. *Biochemical and Biophysical Research Communications, 141*(1), 27-32.

Egner, I. M., Bruusgaard, J. C., & Gundersen, K. (2016). Satellite cell depletion prevents fiber hypertrophy in skeletal muscle. *Development (Cambridge), 143*(16), 2898-2906.

Engelsen, B. (1986). Neurotransmitter glutamate: Its clinical importance. *Acta Neurologica Scandinavica, 74*(5), 337-355.

Estienne, M. J., Harter-Dennis, J. M., Barb, C. R., Hartsock, T. G., Campbell, R. M., & Armstrong, J. D. (1996). N-Methyl-D,L-Aspartate-Induced Growth Hormone Secretion in Barrows: Possible Mechanisms of Action. *Journal of Animal Science, 74*(3), 597-602.

Estienne, M. J., Hurlock, W. F., & Barb, C. R. (1998). Serum Concentrations of Luteinizing Hormone, Growth Hormone, and Cortisol in Gilts Treated with N-Methyl-D,L-Aspartate during the Estrous Cycle or after Ovariectomy. *Journal of Animal Science, 76*(8), 2162-2168.

Estienne, M. J., Schillo, K. K., Green, M. A., Hileman, S. M., & Boling, J. A. (1989). N-methyl-d,l-aspartate stimulates growth hormone but not luteinizing hormone secretion in the sheep. *Life Sciences, 44*(21), 1527-1533.

Ferrini, R. L., & Barrett-Connor, E. (1998). Sex hormones and age: A cross-sectional study of testosterone and estradiol and their bioavailable fractions in

community-dwelling men. *American Journal of Epidemiology, 147*(8), 750-754.
Fimland, M. S., Helgerud, J., Gruber, M., Leivseth, G., & Hoff, J. (2009). Functional maximal strength training induces neural transfer to single-joint tasks. *European Journal of Applied Physiology, 107*(1), 21-29.
Flann, K. L., Lastayo, P. C., McClain, D. A., Hazel, M., & Lindstedt, S. L. (2011). Muscle damage and muscle remodeling: No pain, no gain? *Journal of Experimental Biology, 214*(4), 674-679.
Foy, M. R. (2011). Ovarian hormones, aging and stress on hippocampal synaptic plasticity. *Neurobiology of Learning and Memory, 95*(2), 134-144.
Fraley, G. S., & Ulibarri, C. M. (2002). Long-term castration effects motoneuron size but not number in the spinal nucleus of the bulbocavernosus in the adult male Mongolian gerbil. *Brain Research, 953*(1-2), 265-271.
Furuchi, T., & Homma, H. (2005). Free D-aspartate in mammals. *Biological and Pharmaceutical Bulletin, 28*(9), 1566-1570.
Gay, V. L., & Plant, T. M. (1987). N-methyl-D,L-aspartate elicits hypothalamic gonadotropin-releasing hormone release in prepubertal male rhesus monkeys (Macaca mulatta). *Endocrinology, 120*(6), 2289-2296.
Goldspink, G. (1985). Malleability of the motor system: A comparative approach. *Journal of Experimental Biology, VOL. 115*, 375-391.
Gondin, J., Duclay, J., & Martin, A. (2006). Soleus- and gastrocnemii-evoked V-wave responses increase after neuromuscular electrical stimulation training. *Journal of Neurophysiology, 95*(6), 3328-3335.
Gonzalez, A. M., Hoffman, J. R., Stout, J. R., Fukuda, D. H., & Willoughby, D. S. (2016). Intramuscular Anabolic Signaling and Endocrine Response Following Resistance Exercise: Implications for Muscle Hypertrophy. *Sports Medicine, 46*(5), 671-685.
Gonzalez, A. M., Hoffman, J. R., Townsend, J. R., Jajtner, A. R., Wells, A. J., Beyer, K. S., . . . Fragala, M. S. (2015). Association between myosin heavy chain protein isoforms and intramuscular anabolic signaling following resistance exercise in trained men. *Physiological reports, 3*(1), e12268.
Gotshalk, L. A., Loebel, C. C., Nindl, B. C., Putukian, M., Sebastianelli, W. J., Newton, R. U., . . . Kraemer, W. J. (1997). Hormonal Responses of Multiset Versus Single-Set Heavy-Resistance Exercise Protocols. *Canadian Journal of Applied Physiology, 22*(3), 244-255.
Häkkinen, K., Alen, M., & Komi, P. V. (1985). Changes in isometric force- and relaxation-time, electromyographic and muscle fibre characteristics of human skeletal muscle during strength training and detraining. *Acta Physiologica, 125*(4), 573-585.
Häkkinen, K., Kraemer, W. J., Pakarinen, A., Triplett-McBride, T., McBride, J. M., Häkkinen, A., . . . Newton, R. U. (2002). Effects of Heavy Resistance/Power Training on Maximal Strength, Muscle Morphology, and Hormonal Response Patterns in 60-75-Year-Old Men and Women. *Canadian Journal of Applied Physiology, 27*(3), 213-231.
Häkkinen, K., & Pakarinen, A. (1993). Acute hormonal responses to two different fatiguing heavy-resistance protocols in male athletes. *Journal of Applied Physiology, 74*(2), 882-887.
Häkkinen, K., Pakarinen, A., & Alen, M. (1987). Relationships between training volume, physical performance capacity, and serum hormone concentrations

during prolonged training in elite weight lifters. *International Journal of Sports Medicine, 8*(Suppl 1), 61-65.

Häkkinen, K., Pakarinen, A., Alen, M., Kauhanen, H., & Komi, P. V. (1988a). Daily hormonal and neuromuscular responses to intensive strength training in 1 week. *International Journal of Sports Medicine, 9*(6), 422-428.

Häkkinen, K., Pakarinen, A., Alen, M., Kauhanen, H., & Komi, P. V. (1988b). Neuromuscular and hormonal adaptations in athletes to strength training in two years. *Journal of Applied Physiology, 65*(6), 2406-2412.

Häkkinen, K., Pakarinen, A., Alen, M., & Komi, P. V. (1985). Serum hormones during prolonged training of neuromuscular performance. *European Journal of Applied Physiology and Occupational Physiology, 53*(4), 287-293.

Häkkinen, K., Pakarinen, A., Kraemer, W. J., Newton, R. U., & Alen, M. (2000). Basal concentrations and acute responses of serum hormones and strength development during heavy resistance training in middle-aged and elderly men and women. *Journals of Gerontology-Biological Sciences and Medical Sciences, 55*(2), B95-105.

Hammond, J., Le, Q., Goodyer, C., Gelfand, M., Trifiro, M., & LeBlanc, A. (2001). Testosterone-mediated neuroprotection through the androgen receptor in human primary neurons. *Journal of Neurochemistry, 77*(5), 1319-1326.

Hansen, S., Kvorning, T., Kjær, M., & Sjøgaard, G. (2001). The effect of short-term strength training on human skeletal muscle: The importance of physiologically elevated hormone levels. *Scandinavian Journal of Medicine and Science in Sports, 11*(6), 347-354.

Harman, S. M., Metter, E. J., Tobin, J. D., Pearson, J., & Blackman, M. R. (2001). Longitudinal effects of aging on serum total and free testosterone levels in healthy men. *Journal of Clinical Endocrinology and Metabolism, 86*(2), 724-731.

Hawke, T. J., & Garry, D. J. (2001). Myogenic satellite cells: Physiology to molecular biology. *Journal of Applied Physiology, 91*(2), 534-551.

Hayes, F. J., Seminara, S. B., Decruz, S., Boepple, P. A., & Crowley Jr, W. F. (2000). Aromatase inhibition in the human male reveals a hypothalamic site of estrogen feedback. *Journal of Clinical Endocrinology and Metabolism, 85*(9), 3027-3035.

Heemers, H. V., & Tindall, D. J. (2007). Androgen receptor (AR) coregulators: A diversity of functions converging on and regulating the AR transcriptional complex. *Endocrine Reviews, 28*(7), 778-808.

Herbst, K. L., & Bhasin, S. (2004). Testosterone action on skeletal muscle. *Current Opinion in Clinical Nutrition and Metabolic Care, 7*(3), 271-277.

Holloway, A. C., & Leatherland, J. F. (1997). The effects of N-methyl-D-,L-aspartate and gonadotropin-releasing hormone on *in vitro* growth hormone release in steroid-primed immature rainbow trout, Oncorhynchus mykiss. *General and Comparative Endocrinology, 107*(1), 32-43.

Holtermann, A., Roeleveld, K., Engstrøm, M., & Sand, T. (2007). Enhanced H-reflex with resistance training is related to increased rate of force development. *European Journal of Applied Physiology, 101*(3), 301-312.

Hornberger, T. A., Chu, W. K., Mak, Y. W., Hsiung, J. W., Huang, S. A., & Chien, S. (2006). The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle. *Proceedings of the National Academy of Sciences of the United States of America, 103*(12), 4741-4746.

Inoue, K., Yamasaki, S., Fushiki, T., Okada, Y., & Sugimoto, E. (1994). Androgen receptor antagonist suppresses exercise-induced hypertrophy of skeletal muscle. *European Journal of Applied Physiology and Occupational Physiology, 69*(1), 88-91.

Izquierdo, M., Ibañez, J., González-Badillo, J. J., Häkkinen, K., Ratamess, N. A., Kraemer, W. J., . . . Gorostiaga, E. M. (2006). Differential effects of strength training leading to failure versus not to failure on hormonal responses, strength, and muscle power gains. *Journal of Applied Physiology, 100*(5), 1647-1656.

Jacobs, B. L., You, J. S., Frey, J. W., Goodman, C. A., Gundermann, D. M., & Hornberger, T. A. (2013). Eccentric contractions increase the phosphorylation of tuberous sclerosis complex-2 (TSC2) and alter the targeting of TSC2 and the mechanistic target of rapamycin to the lysosome. *Journal of Physiology, 591*(18), 4611-4620.

Jordan, C. L., Price Jr, R. H., & Handa, R. J. (2002). Androgen receptor messenger RNA and protein in adult rat sciatic nerve: Implications for site of androgen action. *Journal of Neuroscience Research, 69*(4), 509-518.

Kadi, F. (2008). Cellular and molecular mechanisms responsible for the action of testosterone on human skeletal muscle. A basis for illegal performance enhancement. *British Journal of Pharmacology, 154*(3), 522-528.

Kadi, F., Schjerling, P., Andersen, L. L., Charifi, N., Madsen, J. L., Christensen, L. R., & Andersen, J. L. (2004). The effects of heavy resistance training and detraining on satellite cells in human skeletal muscles. *Journal of Physiology, 558*(3), 1005-1012.

Kanai, Y., & Hediger, M. A. (1992). Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature, 360*(6403), 467-471.

Kernell, D., & Hultborn, H. (1990). Synaptic effects on recruitment gain: a mechanism of importance for the input-output relations of motoneurone pools? *Brain Research, 507*(1), 176-179.

Klimstra, M., & Zehr, E. P. (2008). A sigmoid function is the best fit for the ascending limb of the Hoffmann reflex recruitment curve. *Experimental Brain Research, 186*(1), 93-105.

Klossner, S., Durieux, A. C., Freyssenet, D., & Flueck, M. (2009). Mechano-transduction to muscle protein synthesis is modulated by FAK. *European Journal of Applied Physiology, 106*(3), 389-398.

Koyama, H., Sekine, M., Furuchi, T., Katane, M., Nimura, N., Shimamoto, K., . . . Homma, H. (2005). A novel L-glutamate transporter inhibitor reveals endogenous D-aspartate homeostasis in rat pheochromocytoma MPT1 cells. *Life Sciences, 76*(25), 2933-2944.

Kraemer, W. J., Fray, A. C., Warren, B. J., Stone, M. H., Fleck, S. J., Kearney, J. T., . . . Gordon, S. E. (1992). Acute hormonal responses in elite junior weightlifters. *International Journal of Sports Medicine, 13*(2), 103-109.

Kraemer, W. J., Häkkinen, K., Newton, R. U., Nindl, B. C., Volek, J. S., McCormick, M., . . . Evans, W. J. (1999). Effects of heavy-resistance training on hormonal response patterns in younger vs. older men. *Journal of Applied Physiology, 87*(3), 982-992.

Kraemer, W. J., Marchitelli, L., Gordon, S. E., Harman, E., Dziados, J. E., Mello, R., . . . Fleck, S. J. (1990). Hormonal and growth factor responses to heavy resistance exercise protocols. *Journal of Applied Physiology, 69*(4), 1442-1450.

Kraemer, W. J., Staron, R. S., Hagerman, F. C., Hikida, R. S., Fry, A. C., Gordon, S. E., . . . Häkkinen, K. (1998). The effects of short-term resistance training on endocrine function in men and women. *European Journal of Applied Physiology and Occupational Physiology, 78*(1), 69-76.

Kvorning, T., Kadi, F., Schjerling, P., Andersen, M., Brixen, K., Suetta, C., & Madsen, K. (2015). The activity of satellite cells and myonuclei following 8 weeks of strength training in young men with suppressed testosterone levels. *Acta physiologica (Oxford, England), 213*(3), 676-687.

Lagerquist, O., Zehr, E. P., & Docherty, D. (2006). Increased spinal reflex excitability is not associated with neural plasticity underlying the cross-education effect. *Journal of Applied Physiology, 100*(1), 83-90.

Lamanna, C., Assisi, L., Botte, V., & Di Fiore, M. M. (2007). Involvement of D-Asp in P450 aromatase activity and estrogen receptors in boar testis. *Amino Acids, 32*(1), 45-51.

Leenders, M., Verdijk, L. B., Van Der Hoeven, L., Van Kranenburg, J., Nilwik, R., & Van Loon, L. J. C. (2013). Elderly men and women benefit equally from prolonged resistance-type exercise training. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences, 68*(7), 769-779.

Loenneke, J. P., Wilson, J. M., Marín, P. J., Zourdos, M. C., & Bemben, M. G. (2012). Low intensity blood flow restriction training: A meta-analysis. *European Journal of Applied Physiology, 112*(5), 1849-1859.

Lunenfeld, B., Arver, S., Moncada, I., Rees, D. A., & Schulte, H. M. (2012). How to help the aging male? Current approaches to hypogonadism in primary care. *Aging Male, 15*(4), 187-197.

Mackenzie, M. G., Hamilton, D. L., Murray, J. T., Taylor, P. M., & Baar, K. (2009). mVps34 is activated following high-resistance contractions. *Journal of Physiology, 587*(1), 253-260.

Mackey, A. L., Esmarck, B., Kadi, F., Koskinen, S. O. A., Kongsgaard, M., Sylvestersen, A., . . . Kjaer, M. (2007). Enhanced satellite cell proliferation with resistance training in elderly men and women. *Scandinavian Journal of Medicine and Science in Sports, 17*(1), 34-42.

Mansouri, S. H., Siegford, J. M., & Ulibarri, C. (2003). Early postnatal response of the spinal nucleus of the bulbocavernosus and target muscles to testosterone in male gerbils. *Developmental Brain Research, 142*(2), 129-139.

Marshall, P. W. M., McEwen, M., & Robbins, D. W. (2011). Strength and neuromuscular adaptation following one, four, and eight sets of high intensity resistance exercise in trained males. *European Journal of Applied Physiology, 111*(12), 3007-3016.

Marx, J. O., Ratamess, N. A., Nindl, B. C., Gotshalk, L. A., Volek, J. S., Dohi, K., . . . Kraemer, W. J. (2001). Low-volume circuit versus high-volume periodized resistance training in women. *Medicine and Science in Sports and Exercise, 33*(4), 635-643.

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *The Journal of biophysical and biochemical cytology, 9*, 493-495.

McCall, G. E., Byrnes, W. C., Fleck, S. J., Dickinson, A., & Kraemer, W. J. (1999). Acute and chronic hormonal responses to resistance training designed to promote muscle hypertrophy. *Canadian Journal of Applied Physiology, 24*(1), 96-107.

McCarthy, J. J., Mula, J., Miyazaki, M., Erfani, R., Garrison, K., Farooqui, A. B., . . . Peterson, C. A. (2011). Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. *Development, 138*(17), 3657-3666.

McKay, B. R., De Lisio, M., Johnston, A. P. W., O'Reilly, C. E., Phillips, S. M., Tarnopolsky, M. A., & Parise, G. (2009). Association of interleukin-6 signalling with the muscle stem cell response following muscle-lengthening contractions in humans. *PLoS ONE, 4*(6).

Melville, G. W., Siegler, J. C., & Marshall, P. W. M. (2015). Three and six grams supplementation of d-aspartic acid in resistance trained men. *Journal of the International Society of Sports Nutrition, 12*(1), 1-6.

Miller, W. L., & Bose, H. S. (2011). Early steps in steroidogenesis: Intracellular cholesterol trafficking. *Journal of Lipid Research, 52*(12), 2111-2135.

Mitchell, C. J., Churchward-Venne, T. A., Bellamy, L., Parise, G., Baker, S. K., & Phillips, S. M. (2013). Muscular and Systemic Correlates of Resistance Training-Induced Muscle Hypertrophy. *PLoS ONE, 8*(10).

Mitchell, C. J., Churchward-Venne, T. A., Parise, G., Bellamy, L., Baker, S. K., Smith, K., . . . Phillips, S. M. (2014). Acute post-exercise myofibrillar protein synthesis is not correlated with resistance training-induced muscle hypertrophy in young men. *PLoS ONE, 9*(2).

Molina, P. (2013). *Endocrine Physiology* (Fourth ed.). New Orleans, Louisana: McGraw-Hill.

Monier, S., & Le Marchand-Brustel, Y. (1982). Insulin affects only the initiation step of protein synthesis in isolated soleus muscles of lean and goldthioglucose obese mice. *Diabetologia, 23*(2).

Moritani, T., & DeVries, H. A. (1979). Neural factors versus hypertrophy in the time course of muscle strength gain. *American Journal of Physical Medicine, 58*(3), 115-130.

Morton, R. W., Oikawa, S. Y., Wavell, C. G., Mazara, N., McGlory, C., Quadrilatero, J., . . . Phillips, S. M. (2016). Neither load nor systemic hormones determine resistance training-mediated hypertrophy or strength gains in resistance-trained young men. *Journal of Applied Physiology, 121*(1), 129-138.

Nagata, Y., Homma, H., Lee, J. A., & Imai, K. (1999). D-Aspartate stimulation of testosterone synthesis in rat Leydig cells. *FEBS Letters, 444*(2-3), 160-164.

Nagata, Y., Homma, H., Matsumoto, M., & Imai, K. (1999). Stimulation of steroidogenic acute regulatory protein (StAR) gene expression by D-aspartate in rat Leydig cells. *FEBS Letters, 454*(3), 317-320.

Neidle, A., & Dunlop, D. S. (1990). Developmental changes in free D-aspartic acid in the chicken embryo and in the neonatal rat. *Life Sciences, 46*(21), 1517-1522.

Noorkoiv, M., Nosaka, K., & Blazevich, A. J. (2010). Assessment of quadriceps muscle cross-sectional area by ultrasound extended-field-of-view imaging. *European Journal of Applied Physiology, 109*(4), 631-639.

Nordlund, M. M. (2010). Improvements in dynamic plantar flexor strength after resistance training are associated with increased voluntary activation and V-to-M ratio. *Journal of Applied Physiology, 109*(1), 19-26.

O'Connor, R. S., Pavlath, G. K., McCarthy, J. J., & Esser, K. A. (2007). Point:Counterpoint: Satellite cell addition is/is not obligatory for skeletal muscle hypertrophy. *Journal of Applied Physiology, 103*(3), 1099-1102.

O'Neil, T. K., Duffy, L. R., Frey, J. W., & Hornberger, T. A. (2009). The role of phosphoinositide 3-kinase and phosphatidic acid in the regulation of

mammalian target of rapamycin following eccentric contractions. *Journal of Physiology, 587*(14), 3691-3701.

Ondo, J. G., Wheeler, D. D., & Dom, R. M. (1988). Hypothalamic site of action for N-methyl-D-aspartate (NMDA) on LH secretion. *Life Sciences, 43*(26), 2283-2286.

Ostrowski, K. J., Wilson, G. J., Weatherby, R., Murphy, P. W., & Lyttle, A. D. (1997). The effect of weight training volume on hormonal output and muscular size and function. *Journal of Strength and Conditioning Research, 11*(3), 148-154.

Ota, N., Shi, T., & Sweedler, J. V. (2012). D-Aspartate acts as a signaling molecule in nervous and neuroendocrine systems. *Amino Acids, 43*(5), 1873-1886.

Petrella, J. K., Kim, J. S., Cross, J. M., Kosek, D. J., & Bamman, M. M. (2006). Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women. *American Journal of Physiology - Endocrinology and Metabolism, 291*(5), E937-E946.

Petrella, J. K., Kim, J. S., Mayhew, D. L., Cross, J. M., & Bamman, M. M. (2008). Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: A cluster analysis. *Journal of Applied Physiology, 104*(6), 1736-1742.

Pinilla, L., Gonzalez, L., Tena-Sempere, M., Dieguez, C., & Aguilar, E. (1999). Gonadal and age-related influences on NMDA-induced growth hormone secretion in male rats. *Neuroendocrinology, 69*(1), 11-19.

Pohl, C. R., Lee, L. R., & Smith, M. S. (1989). Qualitative changes in luteinizing hormone and prolactin responses to N-methyl-aspartic acid during lactation in the rat. *Endocrinology, 124*(4), 1905-1911.

Prescott, J., & Coetzee, G. A. (2006). Molecular chaperones throughout the life cycle of the androgen receptor. *Cancer Letters, 231*(1), 12-19.

Price, M. T., Olney, J. W., Mitchell, M. V., Fuller, T., & Cicero, T. J. (1978). Luteinizing hormone releasing action of N-methyl aspartate is blocked by GABA or taurine but not by dopamine antagonists. *Brain Research, 158*(2), 461-465.

Raucci, F., D'Aniello, S., & Di Fiore, M. M. (2005). Endocrine roles of D-aspartic acid in the testis of lizard Podarcis s. sicula. *J Endocrinol, 187*(3), 347-359.

Reaburn, P., Logan, P., & Mackinnon, L. (1997). Serum testosterone response to high-intensity resistance training in male veteran sprint runners. *Journal of Strength and Conditioning Research, 11*(4), 256-260.

Remple, M. S., Bruneau, R. M., VandenBerg, P. M., Goertzen, C., & Kleim, J. A. (2001). Sensitivity of cortical movement representations to motor experience: evidence that skill learning but not strength training induces cortical reorganization. *Behavioural Brain Research, 123*(2), 133-141.

Richmond, E. J., & Rogol, A. D. (2007). Male pubertal development and the role of androgen therapy. *Nature Clinical Practice Endocrinology and Metabolism, 3*(4), 338-344.

Riesen, W. F. (1990). How reliable is Reflotron-HDL cholesterol determination. *Schweizerische Medizinische Wochenschrift, 120*(51-52), 1971-1975.

Rochira, V., Zirilli, L., Genazzani, A. D., Balestrieri, A., Aranda, C., Fabre, B., . . . Maffei, L. (2006). Hypothalamic-pituitary-gonadal axis in two men with aromatase deficiency: Evidence that circulating estrogens are required at the hypothalamic level for the integrity of gonadotropin negative feedback. *European Journal of Endocrinology, 155*(4), 513-522.

Rodgers, L. J., Schriefer, J. H. M., Gunnels, T. A., & Bloomer, R. J. (2016). Impact of a Multi-Component Dietary Supplement on Blood Testosterone, Nitrate/Nitrite and Physical Performance in Resistance-Trained Men. *British Journal of Medicine and Medical Research, 11*(1), 1-11.

Rønnestad, B. R., Nygaard, H., & Raastad, T. (2011). Physiological elevation of endogenous hormones results in superior strength training adaptation. *European Journal of Applied Physiology, 111*(9), 2249-2259.

Sale, D. G., Upton, A. R. M., McComas, A. J., & MacDougall, J. D. (1983). Neuromuscular function in weight-trainers. *Experimental Neurology, 82*(3), 521-531.

Santillo, A., Falvo, S., Chieffi, P., Burrone, L., Chieffi Baccari, G., Longobardi, S., & Di Fiore, M. M. (2014). d-aspartate affects NMDA receptor-extracellular signal–regulated kinase pathway and upregulates androgen receptor expression in the rat testis. *Theriogenology, 81*(5), 744-751.

Santtila, M., Kyröläinen, H., & Häkkinen, K. (2009). Serum hormones in soldiers after basic training: Effect of added strength or endurance regimens. *Aviation Space and Environmental Medicine, 80*(7), 615-620.

Sato, T., Nakashima, A., Guo, L., & Tamanoi, F. (2009). Specific activation of mTORC1 by Rheb G-protein in vitro involves enhanced recruitment of its substrate protein. *Journal of Biological Chemistry, 284*(19), 12783-12791.

Schainker, B. A., & Cicero, T. J. (1980). Acute central stimulation of luteinizing hormone by parenterally administered N-methyl-D,L-aspartic acid in the male rat. *Brain Research, 184*(2), 425-437.

Schoenfeld, B. J. (2013). Potential mechanisms for a role of metabolic stress in hypertrophic adaptations to resistance training. *Sports Medicine, 43*(3), 179-194.

Schoenfeld, B. J., Wilson, J. M., Lowery, R. P., & Krieger, J. W. (2016). Muscular adaptations in low- versus high-load resistance training: A meta-analysis. *European Journal of Sport Science, 16*(1), 1-10.

Schott, J., McCully, K., & Rutherford, O. M. (1995). The role of metabolites in strength training - II. Short versus long isometric contractions. *European Journal of Applied Physiology and Occupational Physiology, 71*(4), 337-341.

Shepherd, P. R., Withers, D. J., & Siddle, K. (1998). Phosphoinositide 3-kinase: The key switch mechanism in insulin signalling. *Biochemical Journal, 333*(3), 471-490.

Siegel, A. L., Kuhlmann, P. K., & Cornelison, D. D. W. (2011). Muscle satellite cell proliferation and association: new insights from myofiber time-lapse imaging. *Skeletal Muscle, 1*(1).

Simpson, E. R., Clyne, C., Rubin, G., Boon, W. C., Robertson, K., Britt, K., . . . Jones, M. (2002). Aromatase - A brief overview. *Annual Review of Physiology, 64*(1), 93-127.

Sinha-Hikim, I., Cornford, M., Gaytan, H., Lee, M. L., & Bhasin, S. (2006). Effects of testosterone supplementation on skeletal muscle fiber hypertrophy and satellite cells in community-dwelling older men. *Journal of Clinical Endocrinology and Metabolism, 91*(8), 3024-3033.

Sinha-Hikim, I., Roth, S. M., Lee, M. I., & Bhasin, S. (2003). Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. *American Journal of Physiology - Endocrinology and Metabolism, 285*(1), E197-205.

Smilios, I., Pilianidis, T., Karamouzis, M., & Tokmakidis, S. P. (2003). Hormonal responses after various resistance exercise protocols. *Medicine and Science in Sports and Exercise, 35*(4), 644-654.

Smith, M. J., Keel, J. C., Greenberg, B. D., Adams, L. F., Schmidt, P. J., Rubinow, D. A., & Wassermann, E. M. (1999). Menstrual cycle effects on cortical excitability. *Neurology, 53*(9), 2069-2072.

Sonenberg, N., & Hinnebusch, A. G. (2009). Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets. *Cell, 136*(4), 731-745.

Sower, S. A., Freamat, M., & Kavanaugh, S. I. (2009). The origins of the vertebrate hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–thyroid (HPT) endocrine systems: New insights from lampreys. *General and Comparative Endocrinology, 161*(1), 20-29.

Spiering, B. A., Kraemer, W. J., Vingren, J. L., Ratamess, N. A., Anderson, J. M., Armstrong, L. E., . . . Maresh, C. M. (2009). Elevated endogenous testosterone concentrations potentiate muscle androgen receptor responses to resistance exercise. *Journal of Steroid Biochemistry and Molecular Biology, 114*(3-5), 195-199.

Spinelli, P., Brown, E. R., Ferrandino, G., Branno, M., Montarolo, P. G., D'Aniello, E., . . . D'Aniello, A. (2006). D-aspartic acid in the nervous system of Aplysia limacina: Possible role in neurotransmission. *Journal of Cellular Physiology, 206*(3), 672-681.

Staron, R. S., Karapondo, D. L., Kraemer, W. J., Fry, A. C., Gordon, S. E., Falkel, J. E., . . . Hikida, R. S. (1994). Skeletal muscle adaptations during early phase of heavy-resistance training in men and women. *Journal of Applied Physiology, 76*(3), 1247-1255.

Stocco, D. M. (1998). A review of the characteristics of the protein required for the acute regulation of steroid hormone biosynthesis: The case for the steroidogenic acute regulatory (StAR) protein. *Proceedings of the Society for Experimental Biology and Medicine, 217*(2), 123-129.

Sun, Y., Fang, Y., Yoon, M. S., Zhang, C., Roccio, M., Zwartkruis, F. J., . . . Chen, J. (2008). Phospholipase D1 is an effector of Rheb in the mTOR pathway. *Proceedings of the National Academy of Sciences of the United States of America, 105*(24), 8286-8291.

Terzis, G., Georgiadis, G., Stratakos, G., Vogiatzis, I., Kavouras, S., Manta, P., . . . Blomstrand, E. (2008). Resistance exercise-induced increase in muscle mass correlates with p70S6 kinase phosphorylation in human subjects. *European Journal of Applied Physiology, 102*(2), 145-152.

Terzis, G., Spengos, K., Mascher, H., Georgiadis, G., Manta, P., & Blomstrand, E. (2010). The degree of p70S6k and S6 phosphorylation in human skeletal muscle in response to resistance exercise depends on the training volume. *European Journal of Applied Physiology, 110*(4), 835-843.

Topo, E., Soricelli, A., D'Aniello, A., Ronsini, S., & D'Aniello, G. (2009). The role and molecular mechanism of D-aspartic acid in the release and synthesis of LH and testosterone in humans and rats. *Reproductive Biology and Endocrinology, 7*(120), 1482-1488.

Van Cutsem, M., Duchateau, J., & Hainaut, K. (1998). Changes in single motor unit behaviour contribute to the increase in contraction speed after dynamic training in humans. *Journal of Physiology, 513*(1), 295-305.

Vangsgaard, S., Taylor, J. L., Hansen, E. A., & Madeleine, P. (2014). Changes in H reflex and neuromechanical properties of the trapezius muscle after 5 weeks of

eccentric training: A randomized controlled trial. *Journal of Applied Physiology, 116*(12), 1623-1631.

Vermeulen, A., Kaufman, J. M., & Giagulli, V. A. (1996). Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males. *Journal of Clinical Endocrinology and Metabolism, 81*(5), 1821-1826.

Vermeulen, A., Verdonck, L., & Kaufman, J. M. (1999). A critical evaluation of simple methods for the estimation of free testosterone in serum. *Journal of Clinical Endocrinology and Metabolism, 84*(10), 3666-3672.

Vila-Cha, C., Falla, D., Correia, M. V., & Farina, D. (2012). Changes in H reflex and V wave following short-term endurance and strength training. *Journal of Applied Physiology, 112*(1), 54-63.

Vila-Chã, C., Falla, D., & Farina, D. (2010). Motor unit behavior during submaximal contractions following six weeks of either endurance or strength training. *Journal of Applied Physiology, 109*(5), 1455-1466.

Voigt, M., Chelli, F., & Frigo, C. (1998). Changes in the excitability of soleus muscle short latency stretch reflexes during human hopping after 4 weeks of hopping training. *European Journal of Applied Physiology and Occupational Physiology, 78*(6), 522-532.

Walker, K. S., Kambadur, R., Sharma, M., & Smith, H. K. (2004). Resistance Training Alters Plasma Myostatin but not IGF-1 in Healthy Men. *Medicine and Science in Sports and Exercise, 36*(5), 787-793.

West, D. W. D., Burd, N. A., Staples, A. W., & Phillips, S. M. (2010). Human exercise-mediated skeletal muscle hypertrophy is an intrinsic process. *International Journal of Biochemistry and Cell Biology, 42*(9), 1371-1375.

West, D. W. D., Burd, N. A., Tang, J. E., Moore, D. R., Staples, A. W., Holwerda, A. M., . . . Phillips, S. M. (2010). Elevations in ostensibly anabolic hormones with resistance exercise enhance neither training-induced muscle hypertrophy nor strength of the elbow flexors. *Journal of Applied Physiology, 108*(1), 60-67.

West, D. W. D., Kujbida, G. W., Moore, D. R., Atherton, P., Burd, N. A., Padzik, J. P., . . . Phillips, S. M. (2009). Resistance exercise-induced increases in putative anabolic hormones do not enhance muscle protein synthesis or intracellular signalling in young men. *Journal of Physiology, 587*(21), 5239-5247.

West, D. W. D., & Phillips, S. M. (2012). Associations of exercise-induced hormone profiles and gains in strength and hypertrophy in a large cohort after weight training. *European Journal of Applied Physiology, 112*(7), 2693-2702.

Wilkinson, S. B., Tarnopolsky, M. A., Grant, E. J., Correia, C. E., & Phillips, S. M. (2006). Hypertrophy with unilateral resistance exercise occurs without increases in endogenous anabolic hormone concentration. *European Journal of Applied Physiology, 98*(6), 546-555.

Willoughby, D. S., & Leutholtz, B. (2013). D-Aspartic acid supplementation combined with 28 days of heavy resistance training has no effect on body composition, muscle strength, and serum hormones associated with the hypothalamo-pituitary-gonadal axis in resistance-trained men. *Nutrition Research, 33*(10), 803-810.

Willoughby, D. S., Spillane, M., & Schwarz, N. (2014). Heavy resistance training and supplementation with the alleged testosterone booster NMDA has no effect on body composition, muscle performance, and serum hormones associated with the hypothalamo-pituitary-gonadal axis in resistance-trained males. *Journal of Sports Science and Medicine, 13*(1), 192-199.

Wilson, R. C., & Knobil, E. (1982). Acute effects of N-methyl-DL-aspartate on the release of pituitary gonadotropins and prolactin in the adult female rhesus monkey. *Brain Research, 248*(1), 177-179.

Wu, Y., Zhao, W., Zhao, J., Pan, J., Wu, Q., Zhang, Y., . . . Cardozo, C. P. (2007). Identification of androgen response elements in the insulin-like growth factor I upstream promoter. *Endocrinology, 148*(6), 2984-2993.

You, J. S., Frey, J. W., & Hornberger, T. A. (2012). Mechanical Stimulation Induces mTOR Signaling via an ERK-Independent Mechanism: Implications for a Direct Activation of mTOR by Phosphatidic Acid. *PLoS ONE, 7*(10).

Zammit, P. S., Golding, J. P., Nagata, Y., Hudon, V., Partridge, T. A., & Beauchamp, J. R. (2004). Muscle satellite cells adopt divergent fates: A mechanism for self-renewal? *Journal of Cell Biology, 166*(3), 347-357.

Zanisi, M., & Messi, E. (1991). Sex steroids and the control of LHRH secretion. *Journal of Steroid Biochemistry and Molecular Biology, 40*(1-3), 155-163.

Zehr, E. P. (2002). Considerations for use of the Hoffmann reflex in exercise studies. *European Journal of Applied Physiology, 86*(6), 455-468.

APPENDICES

Appendix 1 – Background Questionnaire

Name: _____ Study ID#:_____

DOB: _____/_____/_____

Age: _____

Date _____/_____/_____

How long have you been doing consistent, structured resistant training?

_____Years _____ Months

Have you taken any testosterone boosting supplements before (including anabolic steroids), and if so which ones?

_____
_____
_____

How long has it been since you have taken these supplements? ___Y____ M

Have you ever had a heart attack, or had heart issues? Yes □ No□

Are you a diabetic? Yes □ No□

Have you ever had any recent major surgeries? Yes □ No□

Had any recent injuries? Yes □ No□

Have you ever had spells of dizziness from exercise? Yes □ No□

Have you ever been diagnosed with hypertension? Yes □ No□

Have you ever been diagnosed with abnormal cholesterol levels?

Yes □ No□

Are you allergic to whey protein or dairy? Yes □ No□

Are you allergic to amino acids? Yes □ No□

Do you have any other know allergies?

_____
_____

PLEASE TURN OVER FOR MORE QUESTIONS ➔

Medications:

Prescription Drug Name	Dosage
Non-Prescription/Supplement Drug Name	**Dosage**

Appendix 2 - Data Sheet – study 1

Name: _____
ID:_____

Height: _____

Sleep time:	_____hrs	(B1)	Weight: _____.____kg
Bench Press 1RM:	_____kg		(B1)
Testosterone:	_____	(B1)	Date: ____/____/____
Estradiol:	_____	(B1)	

Sleep time:	_____hrs	(B2)	Weight: _____.____kg
Bench Press 1RM:	_____kg		(B2)
Testosterone:	_____	(B2)	Date: ____/____/____
Estradiol:	_____	(B2)	

Sleep time:	_____hrs	(B3)	Date: ____/____/____
Testosterone:	_____	(B3)	
Estradiol:	_____	(B3)	

Sleep time:	_____hrs	(B4)	Weight: _____.____kg
Bench Press 1RM:	_____kg		(B4)
Testosterone:	_____	(B4)	Date: ____/____/____
Estradiol:	_____	(B4)	

Sleep time:	_____hrs	(B5)	Weight: _____.____kg
Testosterone:	_____	(B5)	
Estradiol:	_____	(B5)	Date: ____/____/____

Appendix 3 – Participant information sheet, study 1

Project Title: Exploring the short term effectiveness of D-Aspartic Acid in resistance trained men undergoing standardised training.

(Phase 1)

Who is carrying out the study?

PhD student in the School of Science and Health at UWS.

You are invited to participate in a study conducted by PhD candidate Geoffrey Melville (School of Science and Health) under the supervision of Dr Paul Marshall and Dr Jason Siegler.

What is the study about?

The purpose of this study is to investigate the effect of the supplement d-aspartic acid on changes in fasted basal testosterone levels during standardised training. D-aspartic acid is a naturally occurring, endogenous amino acid that is found in nervous and endocrine tissues of the human body. Previous research has suggested that d-aspartic acid has the potential to increase levels of testosterone. The supplement d-aspartic acid will be sourced from supplier Bulk Nutrients and is fit for human consumption. The proposed research will help the investigators better understand the effectiveness of the supplement.

Is there an eligibility criteria?

Two or more years resistance training experience (consistent training excluding de-loading phases); male; 18 – 36 years old; healthy; have the ability to squat at least 130% of their bodyweight; have the ability to bench 100% of their bodyweight; ability

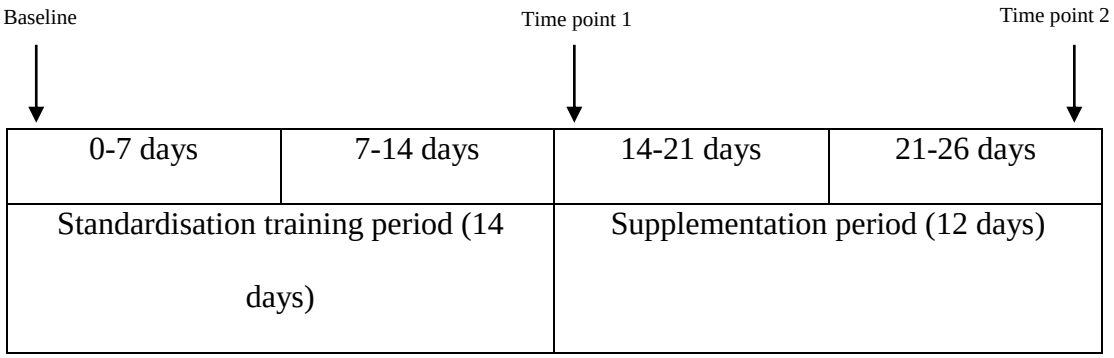
to communicate in English; no acute or chronic medical conditions which would make resistance training hazardous or primary outcome measures impossible to assess; willingness and cognitive ability to provide written informed consent to participate in the trial.

As with any product consumed or brought into contact with humans, there is a risk of developing an allergic reaction. During the study participants assigned to the experimental groups will be consuming the supplement d-aspartic acid. If you have a known allergy to this product or other amino acids, you will also be excluded from the study.

What does the study involve?

The study will require you to attend the research laboratory at UWS Campbelltown campus building 20 on 6-8 occasions. The first session will include a blood draw, one repetition maximum (1RM) bench press, along with familiarisation of the exercise program. In this session you will be given training and nutritional diaries, to be filled out to the best of your ability throughout the study. You will be required to come into the lab in the morning after a 12 hour fast (B1/1RM). After a 5-10 minute resting period, the investigators will take a venous blood sample from the forearm. Once per week for the duration of the study, you can attend the university gym for a supervised training session (if required or need some technique questions answered). You will be expected to train four times per week starting the day of the initial testing session. At the beginning of the experimental period (B2/1RM), you will be randomly assigned a particular dose of the supplement (d-aspartic acid) or a placebo, and you will consume a daily dose your allocated pills for the duration of the experimental period (14 days). You will continue training the same way as during the first 2 weeks. Throughout the

experimental period, further blood draw testing will be conducted, following the same procedures as initial testing and collection (O1-3 & B3-5). The following figure will help illustrate the timeline.



How much time will the study take?

The study will be conducted over 6 weeks. Your involvement will require you to attend the research laboratory on 6-8 occasions and research gym on 4 occasions. The first session will take approximately 60 minutes; gym sessions should take between 60-90 minutes, and blood work collection should be completed within 30 minutes.

Will the study benefit me?

At the end of the study, you will be given access to the results of the study. This will provide you with information about the effectiveness of this particular supplement.

Will the study involve any discomfort for me?

Yes. First, the 1RM testing sessions are designed to find how much weight you are able to move for one repetition, which can be uncomfortable. Second, the sampling of venous blood involves inserting a needle into one of your forearm veins (like a standard blood test). Most people experience this as a brief, transient sharp pain. Third,

the amount of training volume required for the study is designed to match as close as possible the average volume for an advanced trained population. Therefore you may experience some discomfort from Delayed Onset of Muscle Soreness (DOMS).

Are there any risks?

Human research into the product d-aspartic acid is still in early stages, and thus all of the potential risks associated with consumption are unknown. Known side-effects of testosterone supplementation (e.g. anabolic steroids) include increased estradiol, reduced HDL cholesterol (good cholesterol) acne, hair loss, gynecomastia, liver damage, mood changes and atrophy of the testicles. While it is thought that d-aspartic acid is likely to raise basal testosterone, current evidence from humans does not suggest the elevation will be to a level associated with reported side-effects of anabolic steroid use. Anecdotal reports of side-effects associated with d-aspartic acid use must be weighed against the likelihood people are also using a variety of other substances that may induce these side-effects. We do not expect any side-effects to be experienced (e.g. reduced HDL cholesterol, increased acne or increased estradiol). However, we will be monitoring levels of estradiol and unwanted physical changes. Furthermore, if you notice or experience any physical changes that you are not used to, or do not expect, please contact Geoffrey Melville or any of the research team as soon as possible.

How is this study being paid for?

The study is being sponsored by Higher Research Degree funds available to PhD candidates, for the School of Science and Health at the University of Western Sydney. Supplement sponsorship is provided by Bulk Nutrients, TAS, Australia.

Will anyone else know the results? How will the results be disseminated?

All aspects of the study, including results, will be confidential, and only researchers will have access to information on participants. Data collected from you will be presented in undergraduate classes, research seminars, conference presentations, postgraduate theses and/or research publications; under none of these circumstances will your identity be revealed to the audience or the readership. Information collected for this project, or generated by this project may be used for another purpose by the researcher, for which ethical approval will be sought. It may be possible for participants to identify each other. Potentially there may be multiple participants training in the gym at the same time. This, however, will not expose any participant to any greater risk. At no point will personal information or results be disclosed to anyone other the specific participant the results relate to.

Can I withdraw from the study?

Participation is entirely voluntary. You are not obliged to be involved and, if you do participate, you can withdraw at any time without giving any reason and without any consequences.

Can I tell other people about the study?

Yes, you can tell other people about the study by providing them with the chief investigator's contact details. They can contact the chief investigator to discuss their participation in the research project and obtain an information sheet.

What if I require further information?

When you have read this information sheet, PhD candidate Geoff Melville will discuss it with you and answer any questions you might have. If you would like to know more

at any stage, please feel free to contact Geoff Melville g.melville@uws.edu.au, 0426263496, 46203917

If you would like to see the current human research on d-aspartic acid, please refer to the following article:

Topo E, Soricelli A, D'Aniello A, Ronsini S, D'Aniello G. The role and molecular mechanism of D-aspartic acid in the release and synthesis of LH and testosterone in humans and rats. Reproductive Biology and Endocrinology. 2009;7.

What if I have a complaint?

This study has been approved by the University of Western Sydney Human Research Ethics Committee (HREC). The approval number is H10087

If you have any complaints or reservations about the ethical conduct of this research, you may contact the Ethics Committee through the Office of Research Services on Tel +61 2 4736 0229 Fax +61 2 4736 0013 or email humanethics@uws.edu.au.

Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome. If you agree to participate in this study, you may be asked to sign the Participant Consent Form.

Appendix 4 – Participant consent form, study 1

Human Research Ethics Committee
Office of Research Services

University of Western Sydney

Participant Consent Form

This is a project specific consent form. It restricts the use of the data collected to the named project by the named investigators.

Note: If not all of the text in the row is visible please 'click your cursor' anywhere on the page to expand the row. To view guidance on what is required in each section 'hover your cursor' over the bold text.

Project Title: Exploring the effectiveness of a testosterone boosting supplement in advanced trainers undergoing standardised training

I,....., consent to participate in the research project titled: Exploring the short term effectiveness of a testosterone boosting supplement in advanced trainers undergoing standardised training

I acknowledge that:I have read the participant information sheet [or where appropriate, 'have had read to me'] and have been given the opportunity to discuss the information and my involvement in the project with the researcher/s. I understand and acknowledge the risks associated with the consumption of d-aspartic acid and resistance training required for the research.

The procedures required for the project and the time involved have been explained to me, and any questions I have about the project have been answered to my satisfaction.I consent to participating in a 1 month resistance exercise program. I consent to giving 2 blood samples and having my strength assessed during the study.

I consent to consuming a postworkout nutrition containing water, maltodextrin and whey protein isolate at the end of each training session. I understand that during the experimental period (weeks 3-4) participatation involves daily consumption of capsules, which will contain either a placebo, or a testosterone boosting supplement (d-aspartic acid). Furthermore I understand that participation also involves the filling out of training and nutritional diaries to the best of my ability.

I understand that my involvement is confidential and that the information gained during the study may be published but no information about me will be used in any way that reveals my identity. I understand that I may be asked to provide media (radio, internet and newspaper) with information regarding my experience however I am not obliged to do so. I understand that I can withdraw from the study at any time, without affecting my relationship with the researcher/s now or in the future.

Signed:

Name:

Date:

Return Address:

Geoff Melville
Sport & Exercise Science
School of Science and Health
University of Western Sydney
Locked Bag 1797, Penrith South DC, NSW 1797

This study has been approved by the University of Western Sydney Human Research Ethics Committee.

The Approval number is: H10087

If you have any complaints or reservations about the ethical conduct of this research, you may contact the Ethics Committee through the Office of Research Services on Tel +61 2 4736 0229 Fax +61 2 4736 0013 or email humanethics@uws.edu.au. Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome.

Appendix 5 - Participant information sheet – study 2

Project Title: Exploring the effectiveness of D-Aspartic Acid on training adaptation during a 12-week training program

Who is carrying out the study?

You are invited to participate in a study conducted by PhD candidate Geoffrey Melville (School of Science and Health), under the supervision of Dr Paul Marshall and Dr Jason Siegler.

What is the study about?

The purpose of this study is to investigate the effect of d-aspartic acid has on changes in fasted basal testosterone levels from 3 months of training. D-aspartic acid is a naturally occurring, endogenous amino acid that is found in nervous and endocrine tissues of the human body. Previous research has suggested that d-aspartic acid has the potential to increase levels of testosterone. The supplement d-aspartic acid will be sourced from supplier Bulk Nutrients and is fit for human consumption. This study will investigate the effects that elevation of testosterone has on training outcomes following a resistance exercise program. Training outcomes measured will be changes in strength, hypertrophy and adaptation of the peripheral somatic nervous system.

Is there an eligibility criteria?

Two or more years resistance training experience (consistent training excluding de-loading phases); male; 18 – 36 years old; healthy; have the ability to squat at least 130% of their bodyweight; have the ability to bench 100% of their bodyweight; ability to communicate in English; no acute or chronic medical conditions which would make

resistance training hazardous or primary outcome measures impossible to assess; willingness and cognitive ability to provide written informed consent to participate in the trial.

As with any product consumed or brought into contact with humans, there is a risk of developing an allergic reaction. During the study, all participants will be asked to consume a drink of maltodextrin (sugar) and whey protein isolate (dairy protein) for post workout nutrition. Therefore anyone with a known allergy to these products is instantly excluded from the study. During the study participants assigned to the experimental groups will be consuming the supplement d-aspartic acid. If you have a known allergy to this product or other amino acids, you will also be excluded from the study.

What does the study involve?

The study will require you to attend the research laboratory at UWS Campbelltown campus building 20 for three testing time points (which may be 1-2 sessions long depending on time available), and conduct training at the university research gym on multiple occasions over a 3 month period. The first session will involve testing 1 repetition maximum (1RM) for the back squat and the bench press. Initial testing will also involve measurement of the thigh muscle using ultrasound technology and measurement of the peripheral somatic nervous system using electrical nerve stimulation. Blood draws will be taken at 0 weeks, 6 weeks and 3 months. During blood draws participants will be required to come into the lab in the morning (7-10 am) after a 12 hour fast (food, coffee). After a 5-10 minute resting period, the investigator will take a venous blood sample (3-4 tubes) from the forearm (similar to a normal blood test). This will be analysed for hormonal levels (TT, FT, E2, SHBG,

ALB) and HDL cholesterol. For the three days surrounding the blood draw testing day (i.e. day before/day of/day after) participants are required to fill out a 24h food diary (9 total).

For the duration of the study participants will come in for supervised training sessions. The first week participants are expected to come in for the four days to become familiar with each of the training days. From then on participants will continue to training four times per week, and are only required to come in for one supervised session, the other three conducted at their normal gym. At the supervised sessions, participants will be asked to bring in the provided training diary for photocopying (to allow for ongoing data entry).

During the experimental period, participants will consume a dose of pills (11 capsules) containing the supplement (DAA) or a placebo each morning. They will train for 12 weeks, with small changes in the program at the 6-week mark. Participants will also be expected to consume the provided post workout nutrition within 30 minutes of finishing their workout.

How much time will the study take?

The study will be conducted over approximately 3 months. Your involvement will require you to attend the research laboratory or research gym on the previously mentioned occasions. The first session for each time point will involve 1RM testing of the bench press and squat, and provided criteria is met this will be followed by neural testing of the calf muscle. This will take approximately 30-45 minutes for 1RM testing and 90 minutes for the calf protocol. The second session at each time point will involve venous blood draws followed by an ultrasound of the quad and calf. Blood draw takes approximately 20 minutes (allowing for relaxation period), and the ultrasound will

take 30-75 minutes, varying from person to person. There is the option if the participate prefers to complete testing in one day. If this was the case, the order of testing would be blood, ultrasound, 1RMs, and neural stimulation. Furthermore, they would be asked to bring in something they could consume for breakfast to have after the blood and ultrasound been completed. Bear in mind this could take 3-4.5 hours.

Will the study benefit me?

At the end of the study, you will be given access to the results of the study. This will provide you with information about the effectiveness of this particular supplement for you as an individual. Throughout the study post-workout nutrition in the form of powder will be provided free of charge. You're weekly allocation of post workout nutrition will be provided to you when you come to your weekly supervised session. Over 3 months of professional, motivated training, participants should expect to see gains in strength and/or hypertrophy.

Will the study involve any discomfort for me?

Yes. First, the 1RM testing sessions are designed to find how much weight you are able to move for one repetition, which can be uncomfortable. Second, the sampling of venous blood involves inserting a sharp needle into one of your forearm veins (like a standard blood test). Most people experience this as a brief, transient sharp pain. Third, nerve stimulation involves a short electrical stimulus applied to a nerve in your leg. Some experience this as a sharp, but transient pain. Fourth, the amount of training volume required for the study is designed to match as close as possible the average volume for an advanced trained population. Therefore you may experience some discomfort from Delayed Onset of Muscle Soreness (DOMS).

Are there any risks?

Human research into the product d-aspartic acid is still in early stages, and thus all of the potential risks associated with consumption are unknown. Known side-effects of testosterone supplementation (e.g. anabolic steroids) include increased estradiol, reduced HDL cholesterol (good cholesterol) acne, hair loss, gynecomastia, liver damage, mood changes and atrophy of the testicles. While there is evidence to suggest that d-aspartic acid can raise basal testosterone, current evidence from humans does not suggest the elevation will be to a level associated with reported side-effects of anabolic steroid use. Anecdotal reports of side-effects associated with d-aspartic acid use must be weighed against the likelihood people are also using a variety of other substances that may induce these side-effects. We do not expect any side-effects to be experienced (e.g. reduced HDL cholesterol, increased acne or increased estradiol). However, we will be monitoring levels of estradiol, HDL cholesterol and unwanted physical changes. Furthermore, if you notice or experience any physical changes that you are not used to, or do not expect, please contact Geoffrey Melville or any of the research team as soon as possible.

How is this study being paid for?

The study is being sponsored by Higher Research Degree funds available to PhD candidates, for the School of Science and Health at the University of Western Sydney. Supplement sponsorship is provided by Bulk Nutrients, TAS, Australia.

Will anyone else know the results? How will the results be disseminated?

All aspects of the study, including results, will be confidential, and only researchers will have access to information on participants. Data collected from you will be presented in undergraduate classes, research seminars, conference presentations, postgraduate theses and/or research publications; under none of these circumstances will your identity be revealed to the audience or the readership. Information collected for this project, or generated by this project may be used for another purpose by the researcher, for which ethical approval will be sought. It may be possible for participants to identify each other. Potentially there may be multiple participants training in the gym at the same time. This, however, will not expose any participant to any greater risk. At no point will personal information or results be disclosed to anyone other the specific participant the results relate to.

Can I withdraw from the study?

Participation is entirely voluntary. You are not obliged to be involved and, if you do participate, you can withdraw at any time without giving any reason and without any consequences.

Can I tell other people about the study?

Yes, you can tell other people about the study by providing them with the chief investigator's contact details. They can contact the chief investigator to discuss their participation in the research project and obtain an information sheet.

What if I require further information?

When you have read this information sheet, PhD candidate Geoff Melville will discuss it with you and answer any questions you might have. If you would like to know more

at any stage, please feel free to contact Geoff Melville g.melville@uws.edu.au, 0426263496, 46203917

What if I have a complaint?

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If you have any complaints or reservations about the ethical conduct of this research, you may contact the Ethics Committee through the Office of Research Services on Tel +61 2 4736 0229 Fax +61 2 4736 0013 or email humanethics@uws.edu.au.

Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome. If you agree to participate in this study, you may be asked to sign the Participant Consent Form.

Appendix 6 – Participant consent form, study 2

Human Research Ethics Committee
Office of Research Services

University of Western Sydney

Participant Consent Form

This is a project specific consent form. It restricts the use of the data collected to the named project by the named investigators.

Note: If not all of the text in the row is visible please 'click your cursor' anywhere on the page to expand the row. To view guidance on what is required in each section 'hover your cursor' over the bold text.

Project Title: Exploring the effectiveness of d-aspartic acid on training adaptation during a 12-week training program.

I,....., consent to participate in the research project titled: Exploring the effectiveness of d-aspartic acid on training adaptation during a 12-week training program.

I acknowledge that:I have read the participant information sheet [or where appropriate, 'have had read to me'] and have been given the opportunity to discuss the information and my involvement in the project with the researcher/s. I understand and acknowledge the risks associated with the consumption of d-aspartic acid and resistance training required for the research.

The procedures required for the project and the time involved have been explained to me, and any questions I have about the project have been answered to my satisfaction. I consent to participating in a 3 month resistance exercise program. I consent to giving 8 blood samples (5 venous and 3 capillary), having my peripheral somatic nervous system tested (via muscle stimulation), thigh and calf muscle analysed for changes in size and my strength assessed throughout the study.

I understand that participation involves daily consumption of capsules, which will contain either a placebo, or the supplement d-aspartic acid. Furthermore I consent to consuming a post workout nutrition containing maltodextrin and whey protein isolate at the end of each training session.

I understand that my involvement is confidential and that the information gained during the study may be published but no information about me will be used in any way that reveals my identity. I understand that I may be asked to provide media (radio, Internet and newspaper) with information regarding my experience however I am not obliged to do so. I understand that I can withdraw from the study at any time, without affecting my relationship with the researcher/s now or in the future.

Signed:

Name:

Date:

Return Address:

Geoff Melville
Sport & Exercise Science
School of Science and Health
University of Western Sydney
Locked Bag 1797, Penrith South DC, NSW 1797

This study has been approved by the University of Western Sydney Human Research Ethics Committee.

The Approval number is: H10087

If you have any complaints or reservations about the ethical conduct of this research, you may contact the Ethics Committee through the Office of Research Services on Tel +61 2 4736 0229 Fax +61 2 4736 0013 or email humanethics@uws.edu.au. Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome.

Appendix 7 – Venepuncture log book

Venepuncture & Cannulation History – SBHS Sport Science Laboratories

	Date	Subject name	Procedure (Cannulation or Venepuncture)	Any comments/additional details	Staff Initials & Signature
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