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An investigation of endogenous ghrelin and growth hormone-releasing hormone following the consumption of two different relative doses of oral l-arginine

by

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Abstract

Individuals consume the amino acid L-arginine (L-arg) to obtain an anabolic response. However, little is known concerning the mechanism for using an oral dose. The primary purpose of this investigation was to examine the influence of consuming two relative doses of oral L-arg on the growth hormone secretagogues (GHS) endogenous ghrelin and growth hormone-releasing hormone (GHRH) at rest. Thirteen healthy men age 25.1 ± 5.0 yr randomly ingested 0.15 $g \cdot kg^{-1}$ of L-arg, 0.075 $g \cdot kg^{-1}$ of L-arg or a placebo on 3 separate occasions. Plasma hormone levels were measured at rest and up to 3 hrs after ingestion. No significant differences were found in plasma ghrelin or GHRH concentrations between the 3 treatment conditions. These findings indicate that the oral ingestion of L-arg, regardless of dose, does not influence plasma concentrations of ghrelin or GHRH at rest.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

L-arginine (L-arg; 2-amino-5-guanidino-pentanoic acid) has been shown to be a conditionally essential amino acid that was first isolated from lupin seeds in 1886, and later identified as a component of animal proteins (Wu & Morris, 1998). It has been found to exist in relatively high concentrations in seafood, watermelon juice, nuts, seeds, algae, meats, rice protein concentrate, and soy protein isolate (Hou, Yin & Huang, 2008; King, Mainous & Geesey 2008; Wu, Collins & Perkins-Veazie, 2007), but is found to be in low concentration in the milk of most mammals (Davis et al. 1994; Wu & Knabe 1994). Aside from its role in protein metabolism, L-arg is involved in the synthesis of creatine, L-ornithine, L-glutamate as well as protein degradation in the ubiquitin-proteasome pathway (Boger, 2007).

L-arg has been shown to stimulate the secretion of growth hormone (GH), a peptide hormone produced by the anterior pituitary which has been shown to be essential for growth and cell reproduction (Martin, 1986) and has been used clinically for treatment of those with a growth hormone deficiency (GHD) (Boer, Blok & Van der veen, 1995). GH has important metabolic effects on a variety of physiological systems including building and sustaining lean body mass (LBM) and maintaining bone mineral density (BMD) (Tannenbaum, Epelbaum & Bowers, 2003). It is the stimulation of GH and these latter physiological effects that are the primary reasons why individuals ingest L-arg as a supplement.

The basis of the pulsatile release of GH remains unknown, but it is known to be influenced by nutritional, body composition, metabolic, and age-related sex steroid mechanisms, adrenal glucocorticoids, thyroid hormones, and renal and hepatic function in adults (Mericq et al., 1995). Arvat et al. (2001) reported that circulating levels of growth hormone-releasing hormone (GHRH) and ghrelin play a critical role in the secretion of GH. These two hormones in addition to L-arg have been reported to be potent growth hormone secretagogues (GHSs). As a result, it has been suggested that L-arg supplementation would increase circulating levels of GHRH and ghrelin to produce an anabolic response (St. Pierre et al., 2003).

Ghrelin displays strong GH-releasing activity mediated by the activation of the GHS receptor type 1a (GHS-R 1a) (Van der Lely, Tschöp, Heiman & Ghigo, 2004). Van der Lely et al. (2004) stated that ghrelin was part of a molecular regulatory interface between energy homeostasis, glucose metabolism, and physiological processes regulated by endocrine axes such as growth and reproduction. According to Van der Lely et al. (2004) and Veldhuis et al. (2006), it was speculated that ghrelin ensures sufficient amounts of energy are available for GH to stimulate growth and repair and may also work synergistically with L-arg in the secretion of GH.

Van der Lely et al. (2004) has reported that the use of ghrelin agonists may be a more practical method of delivery for an agent that would potentially stimulate endogenous GH since GH is a large protein molecule that must be administered by injection or inhalation. Yin, Li, Xu, An, and Zhang (2009) found

GH therapy in GH deficient patients significantly decreased serum ghrelin concentrations. It is possible that this negative effect on ghrelin secretion could lead to hyperphagia; obesity and other metabolic syndromes; growth retardation; cardiovascular and/or reproduction system disorders; as well as other pathological conditions (Yin et al., 2009). L-arg has been show to be a ghrelin agonist (Veldhuis et al., 2006) and because of this, supplementation may aid in preventing some of these undesirable effects. Based on these findings, higher circulating levels of ghrelin may be beneficial to reverse some of the adverse effects associated with GHD and improve quality of life.

GHRH is required for endogenous pulsatile GH secretion and statural growth (Mericq et al., 1995), as GHRH deficiency in healthy individuals has been found to disrupt the coordinated and orderly release of GH (Veldius et al., 2000). Gianotti et al. (2000) examined the effects of L-arg on the responsiveness to GHRH and GH secretion in humans and discovered that the GH response to GHRH was enhanced by L-arg co-administration. Ghigo et al. (2001) also reported a greater GH response with L-arg supplementation. This research supports the hypothesis that L-arg may influence the action of growth hormone-inhibiting hormone (GHIH) and counteract negative feedback actions exerted by GH as L-arg has been shown to inhibit the secretion of endogenous GHIH from the hypothalamus, resulting in greater release of GHRH (Gianotti et al., 2000). It remains possible that a GHRH agonist such as L-arg supplementation may be a viable way to increase circulating GH. Therefore, L-arg may increase GHRH levels, providing a GH stimulus and subsequent anabolic response.

1.2 Significance of the Study

GHRH and ghrelin are two important hormones that aid in GH secretion and have been found to be influenced by L-arg supplementation (Veldhuis et al., 2006; Ghigo et al., 2001). Determining plasma concentrations of GHRH and ghrelin following relative L-arg dosing may provide new insight into the optimal L-arg dose to increase circulating levels of these GHSs to obtain an anabolic effect. Response to L-arg administration however has a high degree of inter-individual variability and may be altered by training status, sex, age, and diet (Chromiak & Antonio, 2002) as well as the amount ingested. Oral doses high enough to induce a significant anabolic response (e.g., 12 grams or higher) are known to cause stomach discomfort and diarrhea (Chromiak & Antonio, 2002). This thesis used two relative doses of L-arg based on body mass to assist in determining the optimal dose to elicit an anabolic effect without adverse effects.

1.3 Purpose & Hypothesis

The purpose of this study was to investigate plasma ghrelin and GHRH concentrations in response to different relative doses of oral L-arg (0.15 $g \cdot kg^{-1}$ of body mass and 0.075 $g \cdot kg^{-1}$ of body mass) and a placebo control condition. L-arg has been associated with an increase in GHRH through a suppression in GHIH (Wideman et al., 2000). As this is well known, it was unnecessary to analyze plasma GHIH concentrations. Determining circulating levels of GHRH and ghrelin was significant as it provided further insight into the possible synergistic relationship between these GHSs as well as in combination with the GHS L-arg. It

has been shown that L-arg supplementation and an increase in plasma GHRH concentration works synergistically to increase ghrelin concentrations (Koutkia, Canavan, Breu, Johnson & Grinspoon, 2004). Thus it was hypothesized that plasma concentrations of ghrelin and GHRH would increase following the two relative doses of L-arg, with higher plasma concentrations reached following the higher relative dose.

1.4 Delimitations

The participant sample consisted of healthy male subjects, between the ages of 20 and 35 years. Controlling for age, gender and health status reduced variability on hormonal responses (Chromiak & Antonio, 2002).

Prior to each of the three testing conditions, pre-experiment food intake (via 24 hour food diary) and physical activity were controlled. The subjects were involved in all three conditions in a double blinded randomized within subject design during which a placebo, a relative low dose and relative high dose of L-arg was consumed based on the subjects' body mass. To ensure reliability in the blood collection and analyses procedures, the same investigators conducted all tests and the same equipment and protocols were used to collect and analyze all data.

The process of blood analyses involved obtaining a resting blood sample prior to L-arg or placebo consumption. Once the amino acid or placebo was ingested, blood samples were taken again at 30, 60, 90, 120 and 180 min post-consumption. The effect of treatment was measured via this same procedure on 3

separate occasions [following a high relative dose of L-arg ($0.15 g \cdot kg^{-1}$), low relative dose of L-arg ($0.075 g \cdot kg^{-1}$) and placebo]. The variables measured in this study included plasma GHRH and plasma ghrelin.

1.5 Limitations

Limitations within the study included the reliance on volitional adherence to pre-study nutrition and physical activity controls (i.e., ensuring subjects fasted after 10:00 pm the night before each laboratory visit and arrived at the university in a rested state). Moreover, only blood plasma samples were collected to measure hormone responses, therefore it is difficult to make complete interpretations on the absorption, synthesis and degradation of L-arg or that circulating levels of hormones are indicative of a particular physiological response.

1.6 Definitions

1) **L-arginine:** a conditionally essential amino acid synthesized from citrulline by the sequential action of the cytosolic enzymes argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). It plays an important role in cell division, the healing of wounds, removing ammonia from the body, immune function, and the release of hormones.
2) **Growth Hormone:** a peptide hormone that stimulates growth and cell reproduction. It is a 191-amino acid, single chain polypeptide hormone which is synthesized, stored, and secreted by the somatotroph cells within the lateral wings of the anterior pituitary gland.

3) **Growth Hormone-Inhibiting Hormone:** is a peptide hormone produced in the periventricular nucleus of the hypothalamus that regulates the endocrine system and affects neurotransmission and cell proliferation via interaction with G-protein-coupled growth hormone-inhibiting hormone receptors and inhibition of the release of numerous secondary hormones.
4) **Growth Hormone-Releasing Hormone:** a 44-amino acid peptide hormone produced in the arcuate nucleus of the hypothalamus that stimulates growth hormone synthesis and secretion from the anterior pituitary gland.
5) **Ghrelin:** a hormone produced mainly by P/D1 cells lining the fundus of the human stomach and epsilon cells of the pancreas that stimulate appetite. It is also produced in the hypothalamic arcuate nucleus where it stimulates the secretion of growth hormone from the anterior pituitary gland.
6) **Somatotropes:** specialized pituitary cells that synthesize and secrete growth hormone.
7) **Secretagogue:** a substance that causes another substance to be secreted.

CHAPTER 2

REVIEW OF LITERATURE

2.1 L-arginine: Function and Bioavailability

L-arg is a building block for tissue protein synthesis. Visek (1986) reported that approximately 5.4 g of L-arg is absorbed each day in adults who ingest an average North American diet. It is important that L-arg is provided from the diet to support nitrogen balance as well as the growth of both carnivores and young omnivore mammals including rats, pigs and dogs; a dietary deficiency in L-arg however does not affect nitrogen balance in healthy humans (Flynn, Meininger, Haynes, & Wu, 2002). According to Flynn et al. (2002), on the basis of nitrogen balance and growth, L-arg was originally classified as an essential amino acid for young mammals, and as a nonessential amino acid for healthy humans. This definition however, has significant conceptual and practical limitations as nitrogen balance studies do not take into consideration the diverse roles of L-arg metabolites (e.g., creatine) in nutrition and physiology, and are not sufficiently sensitive to fully evaluate dietary requirements of L-arg for metabolic needs by the body (Flynn et al., 2002). Adult mammals fed an L-arg deficient diet exhibited metabolic, neurological, reproductive, and developmental disorders (e.g., hyperammonemia, decreased food intake, muscle tremors, and decreased sperm count by ~90% in adult men) (Flynn et al., 2002). These observations argue that functional needs other than nitrogen balance and growth should be an important criterion for the classification of L-arg as an essential or nonessential

amino acid for mammals, including humans (Flynn et al., 2002). It is now recognized that L-arg is a conditionally essential amino acid for adult humans under such conditions as trauma and burn injury (Flynn et al., 2002).

The structure of L-arg was established by alkaline hydrolysis to yield ornithine and urea in 1897 and by synthesis from benzoyl ornithine in 1910 (Wu & Morris, 1998). Although high activities of arginase (enzyme that hydrolyses L-arg to ornithine and urea) had been identified in the liver in 1904, according to Wu and Morris (1998), it was the discovery of the ornithine cycle (i.e., urea cycle), by Krebs and Henseleit in 1932 that led to the revelation of prominent roles of L-arg in physiology and metabolic pathways. Thus, Wu and Morris (1998) stated that the regulation of L-arg homoeostasis (i.e., balance in dietary L-arg intake, whole-body protein turnover, L-arg synthesis and catabolism) is of considerable nutritional and physiological significance.

Due to a relatively high arginase activity in the small intestinal mucosa, ~40% of dietary L-arg is degraded during absorption and the remainder enters the portal vein (Flynn et al., 2002). Because the amino acid transport system is virtually absent from hepatocytes (i.e., cells of the liver), > 85% of the L-arg delivered to the liver is not taken up by this organ. Thus, digestibility of protein-bound L-arg is 90% in adults, but only ~50% enters systemic circulation (Flynn et al., 2002).

2.2 L-arginine as a Growth Hormone Secretagogue

Amino acids have been identified as potent stimulators of GH secretion. Among them, L-arg has been considered one of the most effective (Adriao et al., 2004). In addition to its role as a precursor for nitric oxide (NO) production and involvement in protein synthesis, exogenous L-arg has been found to act as a secretagogue, increasing GHRH and ghrelin plasma concentrations while inhibiting GHIH secretion, resulting in higher circulating GH (Jones, Borshiem, & Wolfe, 2004; Gianotti et al., 2000).

2.3 GHRH: Function and Distribution

Human GHRH was originally isolated from two pancreatic tumors in patients with acromegaly as 44- and 40- amino acid forms with resting plasma levels of 15 picograms/milliliter (pg/ml) (Giustina & Veldhius, 1998). These bioactive peptides were derived from either of two larger polypeptide precursors (pre-pro GHRH 107 and 108) (Giustina & Veldhius, 1998). According to Giustina and Veldhius (1998), the human GHRH gene resides on chromosome 20 and the naturally occurring variants of GHRH (1-44 and 1-40), along with the synthetic analog (1-29), are biologically equipotent on a molar basis in their capacity to stimulate GH release in humans. The human receptor of GHRH has also been cloned and is a member of the seven-transmembrane-spanning domain G-protein-linked receptor superfamily and is clearly distinguishable from that of ghrelin or GHIH (Giustina & Veldhius, 1998).

Immunoreactive GHRH exists in high concentrations in neurons of the median eminence (ME) and arcuate nucleus (ARCN) of mammalian and nonmammalian species. GHRH is also expressed in the anterior hypothalamic region, the dorsomedial and ventromedial nuclei (DMN and VMN respectively) as well as in the secretory granules and nuclei of somatotropes (Giustina & Veldhius, 1998). Giustina and Veldhius (1998) have stated that hypothalamic GHRH neurons receive significant afferents from GHIH neuronal nuclei, which are hypothesized to be important in the GHRH-GHIH interplay that presumptively directs GH pulsatility.

2.4 GHRH as a Growth Hormone Secretagogue

GHRH has been found to stimulate GH synthesis by increasing the transcription rate of the GH gene. It has also been shown to enhance GH secretion by binding to GH-specific receptors on the membranes of the somatotropes within the pituitary (Giustina and Veldhius, 1998).

GHRH signal transduction to elicit GH secretion involves G_s-protein, adenylate cyclase (isoform II and/or IV), cyclic 3', 5' adenosine monophosphate (cAMP), and protein kinase A (PKA). Intracellular Ca^{2+} concentrations are also increased by GHRH via Ca^{2+} influx (by means of L- and T-type voltage-sensitive Ca^{2+} channels) as well as phospholipase C hydrolysis of phosphatidyl inositol, leading to the mobilization of intracellular Ca^{2+} stores and GH release (Anderson et al., 2004).

Dimaraki and Barkan (1999) attempted to investigate the role of GHRH in GH secretion in humans pharmacologically, as the methodologies used in animals, such as immunoneutralization of GHRH or direct pituitary-portal sampling were impractical. Dimaraki and Barkan (1999) blocked the GHRH receptor using the compound [N-Ac-Tyr^1-, D-Arg^2] GHRH (1-29) NH_2, as it is a specific and selective GHRH antagonist (GHRH-ant) both *in vivo* and *in vitro* to examine the role of endogenous GHRH in the generation of GH pulsatility in humans. The researchers first showed that a single intravenous (IV) bolus dose of GHRH-ant (400 μg/kg) blocked the pituitary response to exogenous GHRH in a time-dependent manner, suppressing the corresponding GH response by 95% at 60 minutes and 4% at 24 hours. The same dose of GHRH-ant suppressed nocturnal GH release by 75%. Failure to more completely suppress nocturnal GH secretion may have been due to either (a) a non-GHRH mechanism accounting for some of the nocturnal GH pulsatility, or (b) waning efficacy of GHRH-ant due to rapid clearance (Dimaraki & Barkan 1999). In a subsequent experiment, Dimaraki and Barkan (1999) administered GHRH-ant as a continuous IV infusion following the loading dose at 2200 h. In this model, nocturnal pulsatile GH secretion in young healthy men was suppressed by almost 90%. These experiments not only confirmed the importance of endogenous GHRH in the generation of pulsatile GH secretion in humans, but also demonstrated that another pathway must exist for GH secretion.

Lesioning of GHRH neurons or GHRH-ant administration was also found to diminish growth length and weight, decrease GHRH-R number and reduce

pituitary GH content in experimental animals (Giustina & Veldhius, 1998). Finally, Giustina and Veldhius (1998) reported that mutations of the extracellular peptide-binding domain of the GHRH-R in the dwarf lit/lit mouse disrupted body growth and abrogated GHRH-R function. GHD has also been found to occur in corresponding mutations of the human GHRH-R gene (Giustina & Veldhius, 1998). Thus, GHRH plays a critical role in GH regulation.

2.5 L-arginine Supplementation Increases GHRH Secretion

Koutkia et al. (2004) reported L-arg to be a potent stimulator of GHRH, and found both L-arg and GHRH to be strong GHSs. Ghigo et al. (2001) stated that L-arg was able to potentiate the GH response to GHRH, making it a powerful and reproducible stimulus of somatotroph secretion. As an example, the GH response to GHRH + L-arg in elderly subjects was preserved and similar to that recorded in young adults and normally growing children (Ghigo et al., 2001). Additionally, Ghigo et al. (1990) reported that L-arg administration in children potentiated GHRH- induced GH increase. These findings agreed with the hypothesis that the GH-releasing effect of L-arg is mediated by suppression of endogeneous GHIH release and an increase in GHRH (Alba-Roth et al., 1988; Ghigo et al., 1990).

A study investigating children and adults with congenital hypopituitarism was conducted to further explore somatotroph responsiveness to L-arg. This study investigated the infusion of GHRH +L-arg as a maximal test to examine the GH-releasable pool in the pituitary. The results demonstrated that the integrity of the

hypothalamus-pituitary connection (evaluated by magnetic resonance imaging-MRI) was essential for GHRH + L-arg to express GH-releasing activity (Ghigo et al., 2001). The study also established that the combined administration of GHRH + L-arg can lead to a massive GH discharge in patients with primary hypothyroidism, in whom the GH response to GHRH alone is impaired (Ghigo et al., 2001).

Gianotti et al. (2000) examined the effects of L-arg (0.5g/kg, IV, from 0-30 min) on the somatotroph responsiveness to GHRH (1μg/kg, IV, at 0 min) and thus GH secretion in humans. They discovered that the GH response to GHRH was strikingly enhanced by L-arg co-administration ($p<0.05$). The authors concluded that L-arg counteracted the inhibitory effect of recombinant human insulin-like growth factor-I (rhIGF-I) on somatotroph responsiveness to GHRH in humans. These findings suggested that the acute inhibitory effect of rhIGF-I on the GH response by GHRH takes place at the level of the hypothalamus, possibly via enhancement of GHIH release, and that L-arg overrides this action (Gianotti et al., 2000).

2.6 Growth Hormone Deficiency (GHD)

It has been clearly demonstrated that adults with GHD have impaired health. GHD in adulthood can lead to impairment in body composition and structure functions (e.g., increased abdominal fat mass, reduced LBM, lowered exercise capacity, impaired cardiac function, reduced BMD), as well as to deranged lipoprotein and carbohydrate metabolism (Aimaretti et al., 1998).

Shortened life expectancy caused by increased cardiovascular morbidity, has also been recorded in hypopituitaric patients with GHD (Aimaretti et al., 1998).

2.7 L-arginine and GHRH: Impact in GHD

Mericq et al. (1995) reported an absence of endogenous GHRH reflexes in most patients with GHD, and tests using GHRH to probe the real secretory capacity of the somatotrope cells in GHD patients are available (Ghigo et al., 1996). Ghigo et al. (1996) stated that L-arg was able to increase the GH response to GHRH in both children and adults with GHD, and L-arg + GHRH tests have been reported to be reliable tests for the diagnosis of primary pituitary GHD in children. Side effects after GHRH administration, when compared to GH therapy, have been found to be extremely mild, consisting of possible transient facial flushing, with no side effects observed after L-arg administration (Ghigo et al., 1996).

Ghigo et al. (1996) reported L-arg + GHRH to be the safest test throughout the adult lifespan and most appropriate for patient compliance. Biller et al. (2002) found the GH response to L-arg + GHRH was independent of age, with less inter and intra-individual variability than with other stimulation tests in GHD patients (e.g., insulin tolerance test-ITT). Ghigo et al. (1996) stated that L-arg was able to restore the reduced GH responsiveness to GHRH in elderly subjects and GHD patients, making it overlap with that in young adults. Maghnie et al. (2001) also reported the GHRH + L-arg test to be one of the most powerful provocative tests of GH secretion.

According to Van Der Klaauw et al. (2006), L-arg was able to increase the pituitary responsiveness to GHRH, inducing greater GH secretion when compared to L-arg supplementation or endogenous GHRH alone. Van Der Klaauw et al. (2006) found that the GH response to L-arg alone was less sensitive to the effects of radiotherapy in GHD patients; GHRH appeared to increase the sensitivity of L-arg and the increase in GH secretion. Importantly, GH tests are contraindicated in patients with severe conditions, such as heart diseases and seizure disorders, and may cause more discomfort than other tests. Thus, Schneider et al. (2006) suggested the use of a GHRH + L-arg test as a safe and sensitive alternative to GH tests (i.e., ITT) in these patients.

2.8 Ghrelin: Function and Distribution

Ghrelin is a natural ligand for the GHS-R and the only peripherally active orexigenic hormone discovered to date. The purified ligand is a peptide of 28 amino acids, in which the serine 3 residue was n-octanoylated. This peptide specifically releases GH both *in vivo* and *in vitro* and the O-n-octanoylation appears to be essential for its activity, at least in terms of GH release (Dieguez & Casanueva, 2000; Shiya et al., 2002). The peptide exists in a form with an acyl side chain attached to the serine 3 residue, which appears vital in binding ghrelin to the GHS-R and to its subsequent orexigenic effects (Murphy, Dhillo & Bloom, 2006).

The human ghrelin gene is located on chromosome 3, precisely at the locus 3p25-26, and consists of 4 exons and 3 introns. The mature protein is

encoded in exons 1 and 2, so that the gene transcript can be processed by alternative splicing to yield two different mature mRNAs and final peptides: ghrelin and des-Gln (14)-ghrelin (Ambrogi et al., 2003). Before being secreted, a portion of ghrelin undergoes esterification in the cytoplasm, which is essential for the biological activity of both forms. Ghrelin is inactivated and biodegraded by plasma proteases and tissue esterases (Ambrogi et al., 2003).

Kojima et al. (2001) tested extracts from different tissues such as brain, lung, heart, kidney and stomach for GHS. The GHS-R was found to be expressed in various hypothalamic and thalamic nuclei, the denate gyrus, substantial nigra, ventral tegmentum, and facial nuclei of the brainstem, implicating a possible central role for ghrelin (Anderson et al., 2004). However, despite many assumptions of the greatest concentrations of GHS-R located in the hypothalamus, surprisingly the highest GHS-R activation was found in stomach extracts (Kojima et al., 2001).

In situ hybridization and immunohistochemical analyses indicated that ghrelin-containing cells are a distinct endocrine cell type found in the submucosal layer of the stomach. These cells, known as P/D_1 cells in humans, contain round, compact, electron-dense granules filled with ghrelin. Normal adult human plasma samples contain 337.2- 404.64 pg/ml of ghrelin (Kojima et al., 2001). According to Kojima et al. (2001), ghrelin is not secreted into the GI tract, but into blood vessels to circulate throughout the entire body; ghrelin immunoreactive cells are also found in the small and large intestines. This indicated that ghrelin secretion,

as opposed to GHRH release, takes place at various sites throughout the body (Kojima et al., 2001).

The accomplishment of Kojima et al. (1999) on the isolation, identification and secretion of ghrelin primarily from both the fundus of the stomach and hypothalamus, has allowed new and intriguing dimensions to arise on the possible overall physiological role of the ghrelin/growth hormone-releasing peptide (GHRP) system. The dual action on GH secretion and food intake, in conjunction with the dual anatomical localization of ghrelin, presented an immediate question about the interdependency of these actions and the site of origin (Bowers, 2001).

Ariyasu et al. (2001) reported that ghrelin mRNA was primarily expressed in the stomach when compared to various other human tissues, as they found a 65% reduction in plasma ghrelin-like immunoreactivity (LI) levels in gastrectomized patients. St. Pierre et al. (2003) also found a 70% reduction in plasma ghrelin-LI levels after gastrectomy in humans, as determined by peptide quantification in the different regions of the gut. Both of these studies confirmed the stomach as the major source of circulating ghrelin in humans.

To expand on this discovery, Ariyasu et al. (2001) examined the effects of fasting and feeding on the plasma ghrelin-LI levels. The study demonstrated that plasma ghrelin-LI levels were significantly elevated after a 12-h fast in humans and rapidly declined within 1 h after habitual feeding. It was suggested that the rise was elicited centrally, and mediated via cholinergic pathways and the vagus nerve to the stomach mucosa (Maier et al., 2004).

The specific physiological signals that regulate ghrelin secretion from the stomach are unknown. Among the known alterations during fasting are the decline in glucose levels, the rise in fatty acid levels, the production of ketones, and several hormonal changes, including the decline in insulin levels and the rise in glucagon, catecholamines, cortisol, and GH levels (Ariyasu et al., 2001).

2.9 Ghrelin as a Growth Hormone Secretagogue

Reverse pharmacology may be an appropriate term to describe the road to ghrelin's discovery as a GHS (Horvath, Diano, Sotonyi, Heiman & Tschop, 2001). According to Horvath et al. (2001), synthetic agonists with ghrelin-like activity (i.e., GHRPs and GHSs) were first discovered by Bowers and co-workers in the late seventies (Bowers, 2001; Momany et al., 1981; Bowers et al., 1980; Bowers, 1998), followed by cloning of the ghrelin-GHS-R in 1996 by Smith and co-workers (Howard et al., 1996; Smith et al., 1996; Smith et al., 1997). Subsequently, the studies by Kojima and co-workers (Kojima et al., 1999; Bowers, 2001; Kojima et al., 2001; Hosoda et al., 2000) led to the identification of an acylated 28 residue peptide as an endogenous bioactive ligand for the GHS-R (Horvath et al., 2001; Ariyasu et al., 2001).

Evidence that synthetic GHSs exhibit strong GH-releasing activity by acting both on the pituitary and hypothalamus where GHS-Rs are present, suggested the existence of another major unknown factor involved in the control of somatotroph function. Thus, the discovery of ghrelin provided additional molecular insights into the physiological signaling mechanisms released by

peripheral tissues connecting the gut to the brain to regulate GH secretion (St. Pierre et al., 2003).

According to Muller et al. (2002), the neuroendocrine mechanism regulating GH secretion appeared to be sensitive and responsive to nutritional state in humans. Fasting was found to rapidly induce an acute and distinct diurnal rhythm to systemic ghrelin concentrations that was not present in the fed state (Muller et al., 2002). Muller et al. (2002) and Tannenbaum et al. (2003) reported that the changes found in serum ghrelin levels during fasting were followed by similar changes in serum GH concentrations, indicating that ghrelin may be the driving force of increased GH secretion during fasting, as well as confirming ghrelin as a critical hormone signal of nutritional status to the GH neuroendocrine axis.

Hartman et al. (1992) investigated the mechanisms underlying augmented serum GH concentrations during nutrient deprivation using a multiple-parameter deconvolution technique and frequent blood sampling. The results demonstrated that endogenous GH secretion rates were enhanced 5-fold by a 2-day fast in normal young men. As ghrelin was found to be mainly produced in a distinct endocrine cell of the stomach (Kojima et al., 2001), this implied that the stomach may exert a direct control over the anterior pituitary (Muller et al., 2002).

Ho et al. (1988) reported that fasting may assist in obtaining a more precise understanding of energy homeostasis and GH secretion, as it would be expected to eliminate the unpredictable effects of mixed nutrients on GH secretion as well as lower IGF-I levels, removing feedback inhibition of GH release. This

open-loop system was predicted to elevate basal GH concentrations and allow identification of spontaneous pulsatility with greater accuracy (Ho et al., 1988). Ho et al. (1988) found that fasting resulted in a progressive increase in mean GH pulse frequency and maximal pulse amplitude, as well as in distinct quantitative and qualitative amplification of the 24-h pattern in GH secretion.

Avram et al. (2005) also discovered that ghrelin can potentiate the pituitary sensitivity to GHRH as well as by itself augment hypothalamic GHRH secretion. According to Hartman et al. (1992), pulses of GH secretion arise from the interactions of GHRH and GHIH released into the hypophyseal-portal circulation. It was speculated that in fasting men, the frequency of GHRH release is increased (due to increased pituitary sensitivity via ghrelin action) and GHIH secretion is prolonged, resulting in increased GH secretory burst amplitude and frequency (Hartman et al., 1992).

Additional evidence of ghrelin as a potent GHS was reported by Kojima et al. (2001) as they further investigated the possible pathways involved in GH regulation. Although GH release from the pituitary was found to be stimulated by hypothalamic GHRH, exogenous GHSs were also thought to induce GH release, but through a different pathway (Kojima et al., 2001). As mentioned, GHRH acts on the GHRH-R to increase intracellular cAMP, which serves as a second messenger to GH release. By contrast, GHSs (i.e., ghrelin) act on a different receptor, increasing the intracellular Ca^{2+} concentration, via 1, 4, 5-trisphosphate signal transduction within the endoplasmic reticulum into the cell cytoplasm, causing depolarization of the cell membrane (Rosicka, Krsek, Jarkovska, Marek,

& Schreiber, 2002). According to Kojima et al. (2001), by screening several rat tissues, a very strong ghrelin activity was found in stomach extracts, requiring only 1 mg of peptide extract to induce a significant Ca^{2+} increase in the GHS-R expressing cells. This occurred due to the activation of the binding ligand-receptor via the phospholipase C signaling pathway, which lead to increased phosphate turnover and protein kinase C (PKC) activation, followed by Ca^{2+} release from intracellular stores and diacylglicerol production (Ambrogi et al., 2003; Ghigo, Arvat, Muccioli & Camanni, 1997).

Kineman and Luque (2007) further investigated the impact of two Ca^{2+} channel blockers on ghrelin as well as GHRH-stimulated GH release in primate pituitary cell cultures. They discovered that the blockade of plasma membrane L-type voltage-sensitive Ca^{2+} channels by nifedipine (1*u*m) to inhibit the influx of extracellular Ca^{2+} completely abolished both ghrelin and GHRH-stimulated GH release, whereas blockade of Ca^{2+} release from intracellular pools by thapsigargin (10 *u*m) abolished GHRH-induced GH release but only blunted the effects of ghrelin (Kineman & Luque, 2007). The results clearly showed that ghrelin may have a more potent effect on GH release comparable to that evoked by GHRH, and therefore provides additional evidence that a direct pituitary action of ghrelin may play a major role in the dynamic release of GH observed in humans and monkeys after ghrelin treatment *in vivo*.

2.10 L-arginine Supplementation Increases Ghrelin Secretion

L-arg is the biological precursor of NO, which has been found to serve as an important signal and effector molecule in humans and animals (Rudovich et al., 2005). Nitric oxide synthase (NOS), an enzyme which catalyzes the generation of NO from L-arg, is present in the oxyntic cells of the stomach, cells in which ghrelin is present. Thus, L-arg may be a possible regulator of ghrelin secretion from oxyntic cells, either by depolarization or via the NO-signaling pathway (Rudovich et al., 2005).

According to Koutkia et al. (2004), Barkan et al. (2003) studied plasma ghrelin concentrations in young healthy men and demonstrated that there was no stimulation of ghrelin during normal saline infusion during the hours of 0800 – 1000 after an overnight fast. Because L-arg is thought to enhance the effects of ghrelin by decreasing GHIH tone, the data suggested potential regulation of ghrelin by changes in endogenous GHIH with L-arg supplementation (Koutkia et al., 2004). Additionally, Koutkia et al. (2004) reported that the stimulation of ghrelin by combined GHRH+L-arg, more than GHRH alone, again suggested potential regulation of ghrelin by L-arg.

Coordinated regulation of ghrelin may be an important component in nutrient signaling to the brain, and ghrelin may be an important physiological regulator of GH. However, further investigation is needed of the pathways by which ghrelin participates in the coordinated regulation of GH (Koutkia et al., 2004).

2.11 L-arginine and Ghrelin: Impact in GHD

At present, the mechanisms of ghrelin action and its interactions with other systems controlling GH secretion remain poorly characterized (Pinilla, Barreiro, Tena-Sempere & Aguilar, 2003). GH-releasing activity of ghrelin has been reported to appear early in the infantile period, is NO dependent and involves a primary hypothalamic site of action (Pinilla et al., 2003; Seoane et al., 2000).

The isolation of ghrelin was reported as one of the most important breakthroughs in the understanding of the regulatory mechanisms involved in the neuroregulation of GH secretion as it gives definitive proof of the existence of a GHS-GHS-R signaling system in the control of GH secretion. Although for many years it became dogma that GH secretion by the anterior pituitary gland was the net result of the antagonistic actions of GHRH and GHIH, a new physiological model of the regulation of GH secretion involving GHRH, GHIH and ghrelin must now be developed. It opens up the possibility of gaining a greater insight into the physiopathological mechanisms involved in the alterations of somatotroph cell function and somatic growth. Finally, it will aid in the development of new agonist and antagonist compounds that may be useful in the treatment of different conditions or disorders (Seoane et al., 2000). Thus, as ghrelin has become a known GHS, determining safe and effective methods to increase its secretion (e.g., L-arg supplementation) may be a possible method to improve or reverse GHD.

2.12 L-arginine Supplementation: Safer Method to Increase GH Compared to GH Therapy

L-arg is a known GHS that is not toxic, and its administration was found to be generally safe for both humans and animals (Fu et al, 2005). In contrast, GH therapy has been reported to have serious side effects, including diabetes, hepatitis and acute renal failure.

When investigating the impact of GH therapy to induce higher circulating GH for the purpose of enhancing athletic performance, Liu et al. (2008) found that GH therapy may in fact worsen exercise capacity. Exercising lactate levels were significantly higher in GH–treated participants than in non–GH treated participants in 2 of 3 studies that evaluated this outcome. Increased exercising lactate levels were associated with decreased exercise stamina and physical exhaustion. While GH therapy resulted in increased use of lipids for fuel during rest [as noted by a statistically significantly lower resting respiratory exchange ratio (RER) and respiratory quotient (RQ)], this improvement did not seem to persist during exercise (Liu et al., 2008).

In another study conducted by Liu et al. (2008), higher rates of adverse events were found in GH–treated participants than in non– GH treated participants, as higher proportions of soft tissue edema, joint pain and carpal tunnel syndrome were found in participants receiving GH therapy. Liu et al. (2008) also reported adverse events relating to fluid retention in GH–treated patients, possibly due to GH's effect on fluid homeostasis. Additionally, GH–

treated participants reported higher rates of fatigue, consistent with the finding that GH may worsen exercise capacity (Liu et al., 2008).

The literature published on randomized, controlled trials evaluating GH therapy in the healthy elderly and individuals with GHD is limited, but suggests that GH therapy is associated with small changes in body composition and increased rates of adverse events (Liu et al., 2007).

According to Carrel, Myers, Whitman and Allen (2002), BMD did increase during 24 months of GH therapy regardless of GH dose. While these longer-term results of GH treatment may be encouraging, a cautious interpretation is still appropriate. Body composition and physical function, while improved, remained significantly abnormal after 4 yr of the GH therapy. Therefore, increasing GH levels by potentiating GHRH and ghrelin plasma concentrations through L-arg supplementation may be a safer approach to obtaining long term benefits of increased GH levels (Carrel et al., 2002).

2.13 Benefit of Higher Circulating GH Levels

GH is secreted in prominent discrete bursts that stimulate somatic growth and mediate certain metabolic adaptations (Moller & Jorgensen, 2009). However, according to Franco, Johannsson, Bengtsson, and Svensson (2006), GH secretion declines with increasing age, leading to metabolic changes seen in normal aging such as an increased relative amount of total and visceral fat and increased low-density lipoprotein cholesterol (LDL-C). Thus, increased circulating GH can stimulate the release and oxidation of FFA, resulting in decreased glucose and

protein oxidation as well as preservation of LBM and glycogen stores (Moller & Jorgensen, 2009). According to Moller and Jorgensen (2009), higher circulating GH resulted in increased protein synthesis and decreased breakdown, decreased amino acid degradation/oxidation and decreased hepatic urea formation; low GH levels were found to increase protein loss and urea production rates by approximately 50%, with a similar increase in muscle protein breakdown. Additionally, Moller and Jorgensen (2009) stated that GH was able to reduce insulin resistance and defend against hypoglycemia to prevent the development of "stress" diabetes during fasting and inflammatory illnesses. Adult patients with GHD are insulin resistant—possibly related to increased adiposity, reduced LBM, and impaired physical performance (Moller & Jorgensen, 2009).

Low GH levels as seen in GHD subjects have been associated with reduced glycerol and FFA release and utilization. Moller and Jorgensen (2009) reported a number of protocols that assessed the effects of higher GH levels during a variety of acute and chronic disease states. In general, the studies showed that the higher GH induced 1) increased lipolysis and elevated FFA levels; 2) protein preservation due to decreased oxidation; 3) elevated levels of IGF-I and insulin; and 4) increased LBM and decreased fat mass. According to Moller and Jorgensen (2009), numerous studies have confirmed the ability of GH to conserve protein and LBM during catabolic illness.

An increase in circulating GH levels have been found to increase physical strength (if combined with moderate exercise), increase BMD, strengthen the immune system, as well as slow the progression of cardiovascular disease (CVD)

by improving an individual's cholesterol profile (Carrel et al., 2002; Franco et al., 2006; Thomas & Monson, 2009). Increased circulating GH levels may therefore result in an improved quality of life.

2.14 Summary

L-arg has been labeled a conditionally essential amino acid and a building block for tissue protein synthesis (Flynn et al., 2002). Aside from its role as a precursor for NO production and involvement in protein synthesis, it has been discovered that exogenous L-arg can also act as a secretagogue, increasing GHRH and ghrelin plasma concentrations, possibly resulting in higher circulating GH levels (Jones, Borshiem, & Wolfe, 2004; Gianotti et al., 2000).

GHRH has been found to increase GH secretion by binding to GH-specific receptors on the membranes of the somatotropes within the pituitary, and L-arg administration has been shown to potentiate the GHRH- induced GH increase (Giustina & Veldhius, 1998; Ghigo et al., 1990).

Additionally, ghrelin has been found to stimulate GH secretion both *in vivo* and *in vitro,* providing additional insight into the signaling mechanisms released by peripheral tissues connecting the gut to the brain to regulate GH secretion; its secretion has also been found to be potentiated by L-arg administration (Dieguez & Casanueva, 2000; Shiya et al., 2002; St. Pierre et al., 2003). The isolation of ghrelin has been reported as one of the most important breakthroughs in the understanding of the regulatory mechanisms involved in the neuroregulation of GH secretion as it gives definitive proof of the existence of a

GHS-GHS-R signaling system in the control of GH secretion (Seoane et al., 2000).

L-arg is a known GHS that is not toxic, and its administration is generally safe for both humans and animals (Fu et al, 2005). Increasing GH levels by potentiating GHRH and ghrelin plasma concentrations through L-arg supplementation may be a safe approach to obtaining long term benefits of increased GH levels (e.g., release and oxidation of FFA, decreased glucose and protein oxidation and preservation of LBM and glycogen stores) (Carrel et al., 2002; Miller & Jorgensen, 2009). Supplementing with L-arg to induce higher circulating GH via GHRH and ghrelin may be considered a possible alternative to GH therapy, however further research is required.

Finally, the isolation of ghrelin and GHRH may provide greater insight into understanding some of the controlling mechanism responsible for the regulation of GH secretion (Dieguez & Casanueza, 2000). If the proposed hypothesis of this study is correct, GHRH and ghrelin may be considered controlling mechanisms of GH release following supplementation with two relative doses of oral L-arg.

CHAPTER 3

METHODS AND PROCEDURES

3.1 Participants and Experimental Design

Fifteen individuals originally volunteered to participate in this study, but two dropped out due to reasons not directly associated with the study. Thus, thirteen individuals completed the study. Inclusion criteria consisted of all participants being male, nonsmokers, healthy (i.e, no major chronic disease or food allergies) and between the ages of 20 and 35 years as minimal fluctuations occur in individuals of this age range; this is also the age in which this supplement is marketed. An orientation meeting was arranged with all participants to provide a thorough explanation of the time commitments and duties in the study and to answer any possible questions regarding the experimental procedures. Potential benefits and risks associated with participation in this study were explained and written informed consent was obtained from each participant (Appendix A). Each participant was provided a list of pretest procedures (Appendix B). The project was approved by a Faculty Research Ethics Board for the use of human subjects. Note that this study was a part of a larger research project in which the effects of different relative doses of L-arg on various physiological variables were investigated (Forbes and Bell, 2010).

The study used a double-blind repeated measures design in which each participant completed all three treatment conditions in a randomized order during each laboratory visit. Both the researcher and the participant were blinded to

which experimental session involved the placebo or different doses of the L-arg supplement. The three treatment conditions were: placebo, 0.075 or 0.15 $g \cdot kg^{-1}$ of body mass of L-arg. The placebo and L-arg supplements were packaged in the same size and type of capsule. The placebo contained a whole wheat flour mixture (Syrotuik et al., 2005) that was similar in color to the L-arg and all capsules were 500 mg.

One day prior to each of the experimental sessions, each participant followed the same dietary intake of food and beverages. This was ensured by having all participants complete a 1-day dietary record to determine the amount of carbohydrate, fat, protein and the total number of calories consumed that was subsequently analyzed using a computer nutritional analysis software program (The Food Processor II for Windows,Version 6.11, ESHA Research, Salem, OR). The nutritional intake of each subject was modified to obtain a 0.8 $g \cdot kg^{-1} \cdot d^{-1}$ of protein in their meals at the same time as maintaining the total calories equal to the subject's original diet record. Each subject was requested to consume this exact diet the day prior to each condition.

3.2 Experimental Protocol

During visits 1-3, subjects were required to arrive at the Exercise Physiology Laboratory at 8:00am for a 3.5 hr period. An intravenous 22 gauge cathelon was inserted into a forearm vein and taped to the forearm by a registered nurse to obtain serial blood samples. Sterile saline (0.9% NaCl) was injected into the cathelon to prevent clotting for subsequent blood samples. A blood sample

was taken at rest (fasted), and at 30, 60, 90, 120, and 180 min after consumption of either a placebo, a high relative dose of L-arg ($0.15 g \cdot kg^{-1}$ of body mass), or a low relative dose of L-arg ($0.075 g \cdot kg^{-1}$ of body mass), which was consumed in capsule form with 500ml of water. Two mls of blood was drawn-off and discarded prior to collecting each sample to make sure no saline was present. The L-arg was a General Nutrition Centers, Inc (GNC) brand and a United States of Pharmacopeia (USP) Certificate regarding the purity and quality of the product was obtained.

Blood samples were collected in a 10-ml tube containing EDTA and kept on ice until centrifuged (refrigerated centrifuge; Damon/IEC Division, Needham Heights, Massachusettes, U.S.A.) for 10 minutes at 1500 xg**.** The plasma was pipetted into separate microcentrifuge tubes and frozen at a temperature of -80 degrees Celsius prior to being analyzed for ghrelin and GHRH concentrations. Figure 8 (Appendix C) illustrates the complete experimental protocol for all three visits to the laboratory.

3.3 Biochemical Analyses

Ghrelin Assay General Principle:

Unacylated ghrelin was measured using an Enzyme Immunoassay (ELISA) kit from SPI-Bio Bertin Pharma (Montigny le Bretonneux, France). Of the two circulating forms of ghrelin (acylated and unacylated), the acylated form is thought to be essential for ghrelin biologic activity, with unacylated ghrelin reported to influence cell proliferation and adipogenesis. Granata et al. (2007)

stated that unacylated ghrelin is the major circulating form of ghrelin. Due to limited funding and the inability to measure both forms of ghrelin, unacylated ghrelin was chosen for the plasma ghrelin analysis in this study. The lower limit of detection (LLD) (i.e., the lowest protein concentration that could be differentiated from zero) was 2.0 $pg \cdot ml^{-1}$. All samples were analyzed in duplicate to further validate measured plasma ghrelin concentrations.

This ELISA was based on a double-antibody sandwich technique (See Figure 2) that is used to detect the presence of an antibody or an antigen in a sample. The sample with an unknown amount of antigen is immobilized on a solid support (usually a microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen in a "sandwich" type procedure). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The antibody is detected by a secondary antibody linked to an enzyme through bioconjugation in this assay.

The procedures required that between each step the plate was washed with a mild detergent solution to remove any proteins or antibodies that were not specifically bound. After the final wash step, the plate was developed by adding an enzymatic substrate to produce a visible signal, which indicated the quantity of antigen in the sample. The wells of the plate were coated with a monoclonal antibody specific to the C-terminal part of the ghrelin. This antibody has been found to bind to any ghrelin introduced into the wells (standard or sample). The acetylcholinesterase (AChE) ghrelin tracer was added to the wells and this

compound recognizes the N-terminal part of unacylated ghrelin. This allowed the two antibodies to form a "sandwich" by binding on different parts of the human unacylated ghrelin.

The conjugate was immobilized on the plate and excess reagents were washed away. The concentration of the human unacylated ghrelin was then determined by measuring the enzymatic activity of the immobilized AChE via spectrophotometry at a wavelength of 415 nm. The intensity of the color was proportional to the amount of unacylated ghrelin present in each well during the immunological incubation (3 hrs at room temperature). Plasma ghrelin concentration was determined mathematically using the slope of the standard curve of known concentrations of ghrelin.

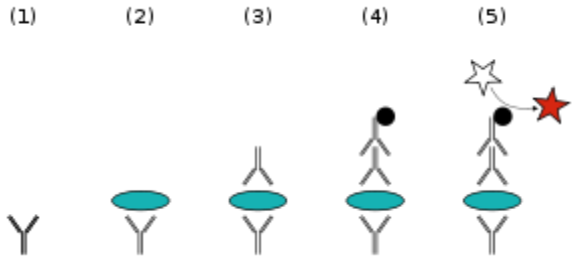

Figure 1. A sandwich ELISA (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form (http://en.wikipedia.org/wiki/ELISA).

The reference control concentration provided with the ghrelin ELISA kit was 25 $pg \cdot ml^{-1}$. After the analysis was complete, the control concentration with each kit was reported as 28.71 ± 1.49 $pg \cdot ml^{-1}$ (expressed as mean ± SEM). The

absorbance due to non-specific binding (NSB) in each well was deducted from the raw absorbance values obtained for the standards and samples. The NSB was considered acceptable by the kit manufacturer if less than 50 milli-absorbance units (mAU); all absorbances obtained in the analysis were below this range. The individual standards were assayed in duplicate to produce the standard curve and the coefficient of determination (r^2) was 0.98. The mean coefficient of variation (CV) for all thirteen duplicate ghrelin samples was 7.76 ± 2.72%. Duplicate blood samples with a CV > 10% (acceptable level of variability of the kit as stated by the manufacturer) occurred as a result of an inadequate blood sample size (this occurred at three time points). One subject had plasma ghrelin concentrations beyond the detectable range of the ghrelin assay kit and as a result was dropped from the study.

GHRH Assay General Principle:

GHRH was measured using an ELISA kit from Uscn Life Science Inc. (Wuhan, China). The LLD for plasma GHRH was 2.5 $pg \cdot ml^{-1}$. Single sample analysis of GHRH was conducted as there was insufficient blood plasma available for duplicate measures.

The microtiter plate provided with this ELISA kit was pre-coated with an antibody specific to GHRH. Standards and samples were pipetted into the appropriate plate wells, with a biotin -conjugated polyclonal antibody preparation specific to GHRH. Avidin was added to each well to bind to the biotin and incubated. Only those wells that contain GHRH, biotin-conjugated antibody and

enzyme-conjugated avidin exhibited a change in color. The enzyme-substrate reaction was then terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of GHRH in the samples was then determined by comparing the optical densities of the samples to the standard curve.

Quality controls were not provided with the GHRH ELISA kit. According to the manufacturer this assay does not require controls to account for cross reactivity with other substances. Additionally, there was no published procedure to account for NSB with this procedure. These omissions must be considered a limitation of this kit. The r^2 for the standard curve was 0.99. GHRH was analyzed using single samples, therefore CV was not determined and no sample CV's were listed in the assay booklet. However, following a plasma GHRH assay conducted by Smith and Brook (1988), intra-assay and inter-assay CV was 6.5% and 9% respectively.

3.4 Data and Statistical Analyses

There were four missing data time points (three following the ghrelin analyses and one following the GHRH analyses) and because of this, they were given the value of the last observation carried-forward (LOCF) (Shao & Zhong, 2003). A two-way ANOVA (3 groups x 6 times) with repeated measures was used to determine whether there were any differences between the different doses and placebo condition over time for the dependent variables (plasma GHRH and ghrelin) using Statistica (StatSoft, Inc., Tulsa, OK). Integrated area under the

curve (iAUC; the cumulative area under the curve) for the 3 hours of sampling was determined for each variable using Graphpad Prism for Windows (GraphPad Software, Inc., CA, USA). A one way repeated measures ANOVA was used to determine if the iAUC was different between conditions (GraphPad Software, Inc., CA, USA). Significant F ratios obtained from the ANOVA's were further analyzed with a Tukey's Honestly Significant Difference (HSD) multiple comparison procedure. Statistical significance was set at $p<0.05$ and results were expressed as means ± standard error of the mean (SEM) unless otherwise noted.

CHAPTER 4

RESULTS

4.1 Subject Recruitment and Attrition

Fifteen individuals volunteered for this study but one dropped out due to reasons not directly associated with the study. An inadequate amount of blood plasma was available to conduct both the plasma ghrelin and GHRH analyses on one participant and so this individual was also removed from the study. Thirteen of the fifteen participants completed the study.

4.2 Subject characteristics

The participants in this study were 25 ± 5 yrs (mean ± SD). Average weight and height was 77.6 ± 8.7 kg and 179 ± 4 cm, respectively (mean ± SD). Average protein intake for the participants in relative and absolute amounts was 1.16 ± 0.21g·kg $^{-1}$ and 90 ± 17 g·d^{-1}, respectively (mean ± SD), with a range of 0.81-1.51g·kg $^{-1}$ (relative) and 64-120 g·d^{-1} (absolute) and a median of 1.14g·kg $^{-1}$ (relative) and 90g·d^{-1} (absolute).

According to Zhello (2006), the Recommended Dietary Allowance (RDA) for protein is 0.8 g·kg $^{-1}$. Thus, some participants in this study were consuming ~35g above the RDA.

4.3 Supplemental L-arginine: Side Effects

Two of the thirteen participants that completed the study experienced adverse side effects following the high dose of L-arg (i.e., GI distress); no negative side effects occurred following the low dose.

4.4 Plasma Ghrelin

A main effect for time was observed for the total plasma ghrelin response with no effect of treatment or interaction effect observed. Ghrelin concentrations at 30 and 60 min were significantly lower than at 180 min ($p<0.05$) (Figure 2). No significant differences were found for ghrelin AUC concentrations when comparing the three treatment conditions (Figure 3).

4.5 Plasma Growth Hormone-Releasing Hormone (GHRH)

No significant differences were found in plasma GHRH concentrations following the three treatment conditions or at any time point (Figure 4). No significant differences were found for GHRH AUC concentrations when comparing the three treatment conditions (Figure 5).

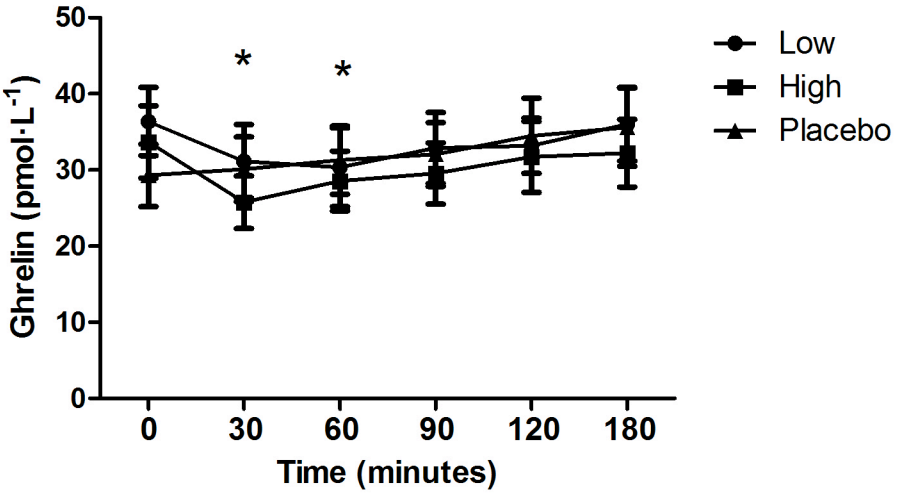

Figure 2. Ghrelin concentrations over time following the three treatment conditions. Values expressed as mean ± SEM, $p<0.05$. *=significantly different from 180 minute time point.

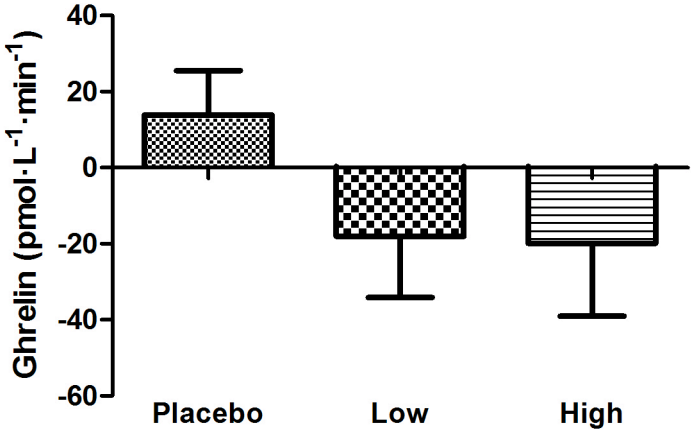

Figure 3. Area under the curve (AUC) values for ghrelin following the three treatments. Values expressed as mean ± SEM, $p<0.05$.

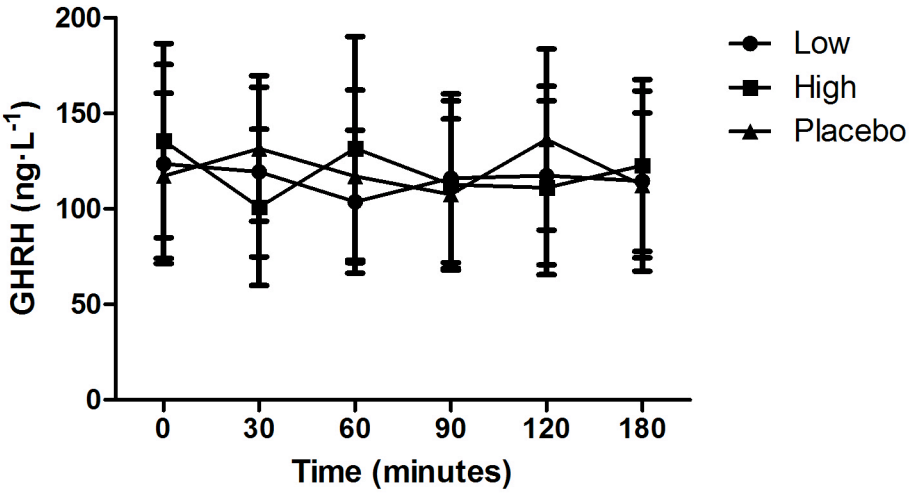

Figure 4. GHRH concentrations over time following the three treatment conditions Values expressed as mean ± SEM, $p<0.05$.

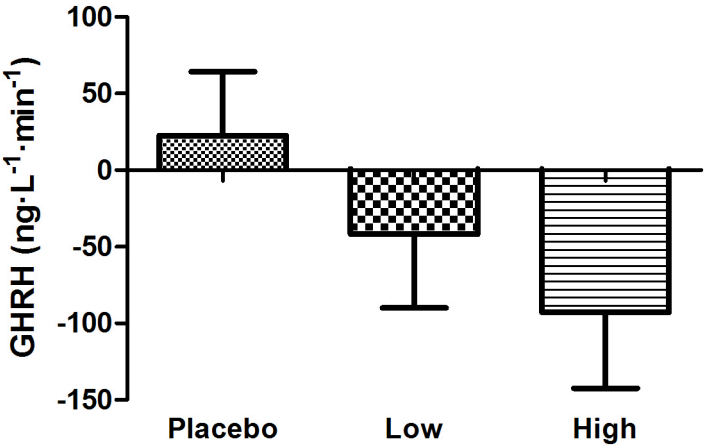

Figure 5. Area under the curve (AUC) values for GHRH following the three treatments. Values expressed as mean ± SEM, $p<0.05$.

CHAPTER 5

GENERAL DISCUSSION & CONCLUSIONS

5.1 Discussion

The purpose of this study was to investigate plasma ghrelin and GHRH response following two different relative doses of oral L-arg (0.150 $g \cdot kg^{-1}$and 0. 075 $g \cdot kg^{-1}$) and a placebo control condition. A significant main effect for time in plasma ghrelin concentration over all treatment conditions was found, but no significant differences in plasma ghrelin or GHRH concentrations occurred between conditions. Thus, the hypothesis for this study stating that plasma levels of ghrelin and GHRH would increase following the consumption of L-arg was rejected. As GHRH and ghrelin are considered controlling mechanisms of GH (Arvat et al., 2001), the results of this study were consistent with the findings of Forbes and Bell (2010) that found no significant differences in GH concentrations despite significantly elevated levels of plasma L-arg in data collected from the same research project. Forbes and Bell (2010) did however note a trend towards increasing GH levels following the L-arg doses, but concentrations did not reach a level necessary to obtain significance. This trend was opposite that of the ghrelin and GHRH levels following 3 hr post treatment; further investigation is required.

5.2 Ghrelin Secretion and L-arginine Ingestion

Not only is the intestinal uptake of L-arg mediated by ghrelin, but L-arg has also been found to enhance ghrelin secretion from oxyntic cells in the stomach (Koutkia et al., 2004). Koutkia et al. (2004) investigated the effect of GHRH

alone and GHRH combined with L-arg on ghrelin secretion. These researchers found that the combination of GHRH and L-arg increased ghrelin to a greater extent than GHRH alone; the physiological mechanism through which GHRH increased ghrelin was not fully clarified. It was suggested that the increase in ghrelin was likely independent of any changes due to extended overnight fast, indicating L-arg as a possible stimulator of ghrelin.

Barkan et al. (2003) also examined L-arg's influence on ghrelin concentration in young healthy men and demonstrated that there was no stimulation of ghrelin during normal saline infusion after an overnight fast. However, because L-arg is thought to enhance the effects of GHRH by decreasing GHIH tone, Barkan et al. (2003) suggested that potential regulation of ghrelin occurs through changes in endogenous GHIH, as GHRH and ghrelin have been found to work in a synergistic manner. As previously noted, Koutkia et al. (2004) showed that the increase in ghrelin concentration with combined GHRH and L-arg was greater than stimulation with GHRH alone, which suggested that the ghrelin release was at least partially regulated by L-arg.

Ghrelin is involved in stimulating somatotroph cells by activating two major signaling cascades involving inositol phosphate and cAMP. It is known that NO (L-arg is the precursor for NO) and its mediator cGMP contribute substantially to the response of somatotrophs to key regulatory hormones, including GHRH, GHIH and leptin (Rodríguez-Pacheco et al., 2008). Rodríguez-Pacheco et al. (2008) investigated the possible role of this signaling pathway in ghrelin. Accordingly, cultures of pituitary cells from prepubertal female pigs were

challenged with ghrelin in the absence or presence of activators or blockers of key steps of the NOS/NO/guanylate cyclase/cGMP route and found NO contributed critically to ghrelin action in somatotrophs (Rodríguez-Pacheco et al., 2008).

In the present study, circulating levels of ghrelin were significantly lower 30 and 60 minutes after ingestion regardless of treatment compared to the 180 minute time point. There were no differences between the two relative doses of L-arg consumed (0.075 versus 0.150 $g \cdot kg^{-1}$). It is important to note these two doses were able to significantly elevate circulating L-arg concentrations in the blood in the same study (Forbes & Bell, 2010). According to Boger and Bode-Boger (2001), it is possible that a fraction of an oral L-arg dose may be metabolized pre-systemically, excreted from the gut or possibly result in a "spillover" into urine from the extremely high plasma concentrations reached. Absolute doses consumed in this study were 11.63 ± 1.3g of L-arg (high dose) and 5.82 ± 1.3g of L-arg (low dose; mean ± SD). It remains possible that these oral doses of L-arg consumed were not high enough to meet a critical threshold to elevate ghrelin levels. However doses of L-arg greater than 12g per day have also been found to cause GI distress (Collier, Casey & Kanaley, 2005) suggesting that doses in high concentrations may not be practical.

Another possible explanation for the lack of change in plasma ghrelin levels from the oral consumption of L-arg may have been due to the methodology of the blood sampling procedures utilized in this study. It has been recommended that the treatment of plasma with aprotinin (protease inhibitor) when preparing the blood samples would improve the accuracy of this analysis. The blood samples

were not treated with aprotinin, however they were kept cold and on ice, thereby reducing this criticism. According to Kojima and Kangawa (2004), it is recommended that the plasma fraction of each blood sample is treated with 1/10 volume of 1 N HC1 following centrifugation to keep samples stable for at least 6-12 months. This was not a requirement of the ELISA assay used to measure ghrelin in this study, however the omission of this treatment may have caused some degradation of ghrelin. Despite the possible methodological limitations of this study, the lack of change in ghrelin concentrations are supported by Forbes and Bell (2010) who reported no significant changes in plasma GH concentrations in the same study.

5.3 GHRH Secretion and L-arginine Ingestion

In addition to its role as a precursor for NO production and protein synthesis, L-arg can act directly as a secretagogue, stimulating GHRH release from the somatotrophs (Ghigo et al., 2001). Ghigo et al. (1990) conducted a study which showed that in children, L-arg administration can potentiate GHRH-induced GH increase and these findings agree with the hypothesis that the GH-releasing effect of L-arg was mediated by suppression of endogeneous GHIH release and an increase in GHRH (Alba-Roth et al., 1988; Ghigo et al., 1990). According to Fisker et al., (1999), most studies suggest that an inhibition of GHIH secretion is responsible for this effect of enhanced GHRH release.

In the present study, neither the high or low relative dose of L-arg administered (0.150 $g \cdot kg^{-1}$ and 0.075 $g \cdot kg^{-1}$ respectively) were able to elicit a

significant change in GHRH concentrations, however as previously mentioned, plasma concentrations of L-arg were significantly elevated in this study (Forbes and Bell, 2010). The lack of GHRH response in this study may reflect the conflicting results of L-arg's influence on GHRH reported in the literature. In one *in vitro* study, NO suppressed GHRH action in rat male pituitary cells in culture, whereas in another *in vitro* study, a decrease in NO production increased GHRH action (Boger & Bode-Boger, 2001). Additionally, Jaffe et al. (1996) stated that L-arg promoted the secretion of hypothalamic GHRH necessary to stimulate GH release and produce spontaneous GH pulses, as well as inhibited GHIH release. These findings were confirmed from direct pituitary portal blood sampling in rats and sheep as the administration of a GHRH-ant attenuated the GH response by 70-90% (Jaffe et al., 1996). Thus, Jaffe et al. (1996) data confirmed that endogenous GHRH plays a major role in the generation of pharmacologically induced GH release. The lack of change in plasma GHRH concentrations reported in this study are again supported by the lack of change in plasma GH in this same study (Forbes & Bell, 2010).

5.4 Limitations

The results of the present study were specific to a healthy male population so the ability to generalize the hormonal changes from L-arg supplementation to women is limited. According to Makovey et al. (2007), women have higher plasma ghrelin levels than men as gender differences have been found to influence the association of fat mass and body size on plasma ghrelin levels. In

women, plasma ghrelin correlated inversely with BMI, total fat mass and fat mass/lean mass ratio, whereas in men, associations with abdominal fat mass and fat distribution index and ghrelin concentrations were observed. Qu et al. (2005) also reported gender differences in basal GHRH levels as the female to male ratio of mean GH responses to GHRH and L-arg was reduced from 2.4 to 1.6. This discrepancy may be due to changes in gender responses to the GHRH or the L-arg, however, further investigation would be required to confirm this.

The blood plasma was stored for > 3 months and some of the plasma underwent freeze/thaw cycles prior to the measurement of the current variables, making this a possible limitation of the study. As a result, it is likely that the integrity and bio-availability of the blood plasma may have been altered. Additionally, because of insufficient blood plasma available to perform a duplicate analysis of GHRH, it was difficult to assess the variability of this assay.

Only plasma samples were used to measure hormonal responses, which made it difficult to make clear interpretations of a physiological response since the concentration at time of measurement is based on release, circulation, binding and removal. The circulating concentration of L-arg is also influenced by absorption, synthesis, and degradation. Additionally, both acylated and unacylated (i.e., total) ghrelin should have been analyzed to determine the total plasma concentration of ghrelin but only unacylated ghrelin was measured in this study.

Most participants in this study were accustomed to consuming more than the RDA of protein for their age (i.e, 0.8 $g \cdot kg^{-1} \cdot d^{-1}$ for males 19 yrs and older) (Zello, 2007) however instructed to only consume the RDA. As a result, it was

possible that the participants consuming >0.8 $g \cdot kg^{-1} \cdot d^{-1}$ on a regular basis had higher daily plasma L-arg concentrations, and thus a diminished sensitivity to the amino acid supplementation.

5.5 Conclusions

The present study investigated plasma ghrelin and GHRH responses following two relative doses of oral L-arg. A main effect for time was found in plasma ghrelin concentrations over the three treatment conditions ($p<0.05$) but no significant differences were found between conditions. There were no significant findings in plasma GHRH concentrations. Since ghrelin and GHRH are potent stimulators of GH (Arvat et al., 2001; Kojima et al., 2001), the lack of significant changes in these variables are supported by other findings from the same study that showed no significant changes in plasma GH levels despite significantly elevated plasma L-arg concentrations (Forbes & Bell, 2010). Based on the present study, the two relative doses of L-arg via oral ingestion were not sufficient to stimulate increases in ghrelin and GHRH.

5.6 Practical Applications

The lack of change in ghrelin and GHRH plasma concentrations following the different relative doses of L-arg consumed suggests that the doses given may have been inadequate to induce significant changes, and perhaps a larger dose of L-arg may have been able to elevate ghrelin and GHRH. However, high oral doses of L-arg are known to cause gastro-intestinal distress. Perhaps IV method of

delivery may be the optimal route of administration to result in greater absorption of L-arg to stimulate higher plasma GHRH concentrations. IV infusion however would not be the optimal method of L-arg delivery to stimulate higher levels of plasma ghrelin as this hormone is found in oxyntic cells in the stomach and thus released from the gut (Koutkia et al., 2004) nor practical as a method of supplementing in athletes.

Although numerous published studies have investigated the relationship between ghrelin and GHRH on GH release, this study was somewhat novel in relation to plasma ghrelin and GHRH hormonal responses following two relative oral doses of L-arg in a single study. The findings of this study suggest that oral doses of L-arg utilized may not stimulate plasma concentrations of GHS's ghrelin and GHRH to a threshold level to induce an anabolic response. A higher oral dose L-arg (i.e., > 12g) may have stimulated a ghrelin and GHRH response but such doses have been shown to cause GI distress.

5.7 Future Directions Related to L-arginine Supplementation, Ghrelin and GHRH

The mechanism(s) of action underlying the GH-releasing effect of ghrelin has yet to be fully clarified. Although studies in animals and humans have indicated that it acts on the pituitary, mainly at the hypothalamic level via enhancement of the activity of GHRH-secreting neurons, further investigation is required (Broglio et. al., 2002).

Coordinated regulation of ghrelin and GH may be an important component

in nutrient signaling to the brain, and ghrelin may be an important physiological regulator of GH (Arvat et al., 2001). Increases in GH may also evoke a negative feedback resulting in the inhibition of ghrelin release. Further investigation will be necessary to examine the pathways by which ghrelin participates in the coordinated regulation of GH (Koutkia et al., 2004). Studies that have examined the influence of L-arg supplementation on ghrelin secretion have also been contradictory. Additional work will be necessary to obtain a better understanding of the physiological impact of L-arg ingestion on the GH control mechanism of ghrelin.

The exact physiological mechanisms through which GHRH secretes GH have not been established in humans, as direct measurements of hypothalamic GHRH output have only been conducted in animals studies (Jaffe et al., 1996). Significant species-specific differences in GH neuro-regulation make the extrapolation of animal data to human physiology questionable; further investigation is required (Jaffe et al., 1996).

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APPENDIX A

Informed Consent

The effects of a low and high dose of L-arginine supplementation on physiological responses at rest.

Principal Investigator(s): Amanda McCarthy, M.Sc. Student, Faculty of P.E. and Rec. 492-7394.

Co-Investigator(s): Dan Syrotuik, Ph.D., Faculty of P.E. and Rec. 492-2018.

Do you understand that you have been asked to be in a research study?	Yes	No
Have you read and received a copy of the attached Information Sheet	Yes	No
Do you understand the benefits and risks involved in taking part in this research study?	Yes	No
Have you had an opportunity to ask questions and discuss this study?	Yes	No
Do you understand that you are free to refuse to participate, or to withdraw from the study at any time, without consequence, and that your information will be withdrawn at your request?	Yes	No
Has the issue of confidentiality been explained to you? Do you understand who will have access to your information?	Yes	No

This study was explained to me by: _____

I agree to take part in this study:

_____	_____	_____
Signature of Research Participant	Date	Witness
_____		_____
Printed Name		Printed Name

I believe that the person signing this form understands what is involved in the study and voluntarily agrees to participate.

Signature of Investigator or Designee	Date

APPENDIX B

Participation Information Form

The effects of a low and high dose of L-arginine supplementation on physiological responses at rest.

Principal Investigator: Amanda McCarthy, M.Sc. Student, Faculty of P.E. & Rec. 492-7394.
Co-Investigator: Dan Syrotuik, Ph.D., Faculty of P.E. & Rec. 492-2018.

Dear Participant.

I (Amanda McCarthy) am a graduate student in the Faculty of Physical Education and Recreation under the supervision of Dr. Dan Syrotuik. I am conducting a study that is researching the effect of a nutritional supplement (the amino acid, L-arginine) on hormones that can be measured in your blood (i.e., ghrelin and growth hormone-releasing hormone). L-arginine is an amino acid (building block of protein) and is found in common foods that you eat (nuts, seeds, dairy products, etc.) and can also be purchased at health food stores. The purpose of this study is to determine the effect of different amounts of L-arginine ingestion on the things in your blood that are affected by consuming this amino acid (note that these are the things listed above).

To be a subject in our study, you must be a healthy male and free of any medical condition (heart disease, diabetes, herpes, liver, kidney etc.) or any type of food allergy since these may influence our results. You must also be able to swallow capsules. These things will be checked with you verbally by one of the

researchers. You will be asked to attend a meeting in the exercise physiology lab (directions will be provided) during which all the procedures will be completely explained to you and allow us to answer any questions you have related to the study. At this meeting we will also talk about and show you all the procedures and how to complete a 1-day record of what you eat over 24 hours. You will be asked to take this form home and complete it on your own time over a typical day but we will ask you to refrain from any alcohol consumption during this day. When finished we will ask that you fax or drop this form off at the lab so we can analyze it and modify it by specifically adjusting the amount of protein in your diet to the recommended amount (15% of total calorie intake) by Health Canada if necessary to make sure that your diet is nutritionally sound. Then we will return this form to you and ask you to eat the same foods that you listed on the form that you eat, the day before each of the 3 experimental trials.

At the orientation meeting, we will measure your height, weight, record your age, and ask you to complete a brief physical activity form. Then each of the 3 experimental trials will be randomly ordered which means that the order that you do them may or may not be the same for everyone. The day before each trial we will ask you to eat the same meals as on your diet record form. Then we will ask that you do not eat after 10:00 pm the night before each trial, but you can drink as much water as you require. The next day, we ask that you come to the exercise physiology lab between 7:00 and 8:00 am to begin each experiment. We ask that you do not exercise (ride your bike to the University for example) before you come as we need to take a fasted, rested blood sample. A small sterile tube

called a cathelon will then be placed into a blood vessel in your forearm with a needle. This is similar to what is used if you have had an "IV" in a hospital or when you have donated blood at Blood Services. This means that we only have to "poke" you once with a needle to do the experiments since the needle will be removed in this procedure and only the soft tube remains to take the blood samples from. This tube has a cap on top and is taped to your arm during the experiment. The nurse will put a small amount of sterile saline (water with some "electrolytes") into the tube to keep it ready for subsequent blood samples. We will take 1 blood sample at rest (fasted), and again after 30, 60, 90, 120, and 180 minutes. This is 6 samples and totals approximately 4 tablespoons (60 ml's) of blood for each experimental trial which does not present any risk to your health at all.

Depending on the random assignment, you will eventually do each of the 3 experimental conditions described below. You will get at least 4 days off in between the bouts.

Trial A- we will give you some "placebo" capsules that simply contain whole wheat flour that you will swallow with 500 ml of water.

Trial B- will provide you again with the 500 ml's of water to consume a certain number of L-arginine capsules that will be calculated based on your body weight (0.075 grams per kilogram body weight of L-arginine).

Trial C- will be similar to Trial B except that more (0.15 $g \cdot kg^{-1}$) capsules of L-arginine will be consumed with water.

Note that we will provide you with a schedule of all your required visits to the lab and these will be as flexible as possible to suit your personal lives.

Risks: The blood samples are performed with sterile equipment but there is a small risk of infection at the site if not properly cared for. However, sterile procedures, cleanliness and use of a band-aid greatly minimize this risk. A registered nurse using standard procedures will conduct the blood sample procedures using the cathelon. Some people feel faint or lightheaded when giving blood samples. If this happens, we will stop the procedure immediately and treat you with standard care. Taking L-arginine may cause an upset stomach, diarrhea, lowered blood pressure, headache, and may increase blood sugar levels in some individuals.

Qualified personnel under the supervision of Dr. Gordon Bell will administer the testing. Personnel are trained to handle identifiable risks and certifications can be produced upon request. The researchers will continuously watch for adverse symptoms and will stop any procedure if at any time they are concerned about your safety. You can also stop any procedure at any time. Please inform the researcher of any of the above- mentioned symptoms experienced during or after the tests.

Benefits: The major benefit of your participation in this study will be to help the researchers understand the nature of L-arginine with various amounts given on certain responses in the body. As a participant you will be provided with a written

report of your personal results if you want. If you are interested in the future research outcomes of this study, you may contact one of the researchers for this information as well.

Total Time Commitment:

Orientation meeting	~30 minutes
Completing diet record at home	~30 minutes (total time)
3-experimental trials	~3×3.5 hours ~10.5 hours
Total testing time =	***~11.5 hours***

Testing time does not include travel to and from the lab.

Confidentiality: To ensure confidentiality and anonymity, personal information will be coded and stored in a file cabinet in a locked office to which only the investigators have access. There will be no way to identify individuals in results that may be published in any report or article. Normally, information is retained for a period of 5 years post publication, after which it may be destroyed. The blood and urine samples are stored in freezers for the same period of time after which they will also be destroyed in biohazard waste. The data will hopefully be presented at a research conference and possibly published in a scientific journal.

Freedom to withdraw: For the purpose of the study you are required to participate in all the procedures but you can withdraw at any time without consequence by simply informing one of the investigators verbally, phone call or email. If you decline to continue or withdraw from the study, all information will

be removed from the study upon your request. Contacting either Amanda McCarthy or Dr. Dan Syrotuik at anytime during the study can do this.

Additional contacts: If you have concerns about the study and wish to speak with someone who is not involved with this study, please call Dr. Wendy Rodgers, Chair of Research Ethics Board, Faculty of Physical Education and Recreation at 492-5910.

Thank you,_____ _____

Amanda McCarthy, M.Sc. **Dan Syrotuik, Ph.D.**

APPENDIX C

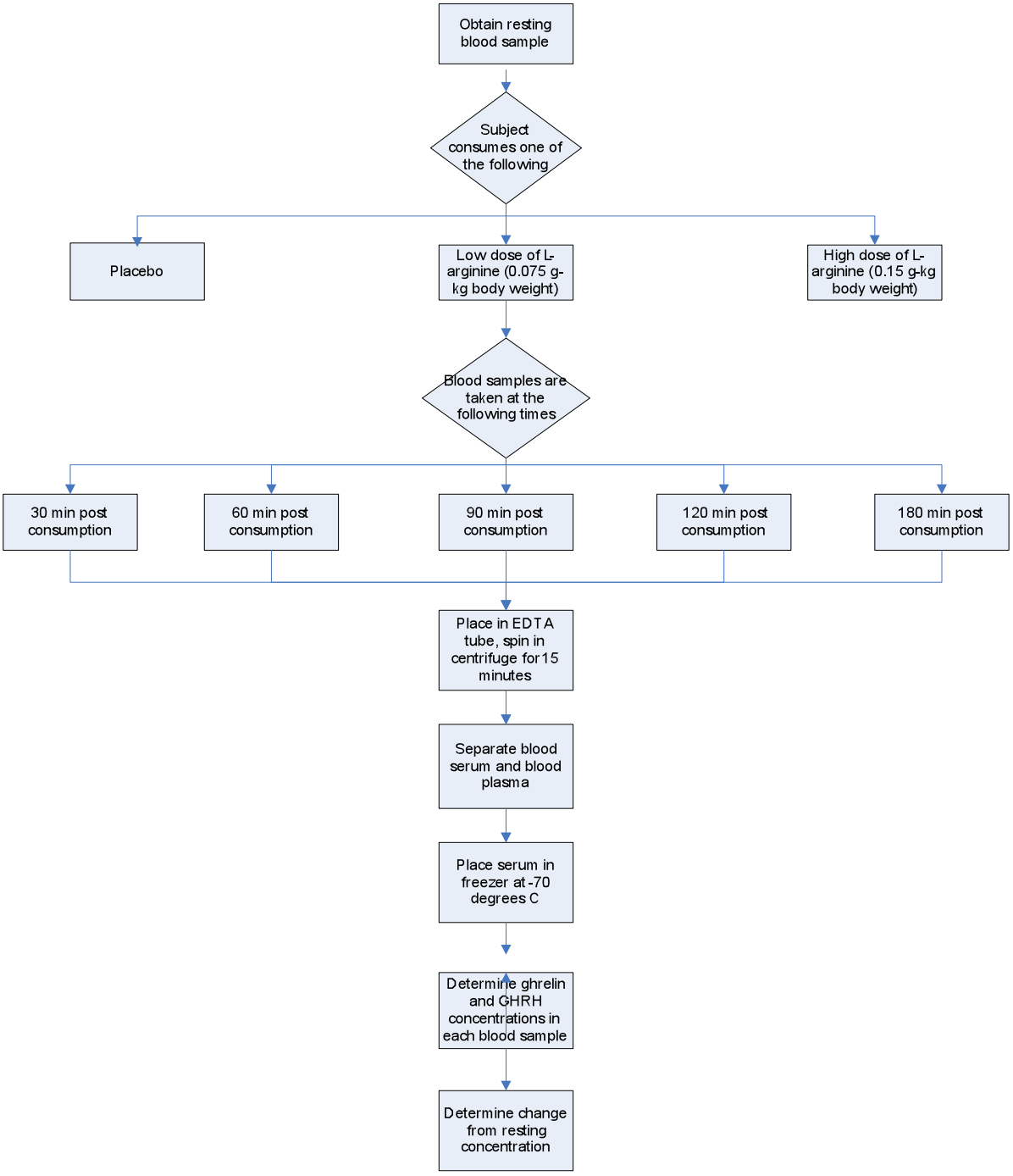

Figure 6. Experimental procedures

APPENDIX D

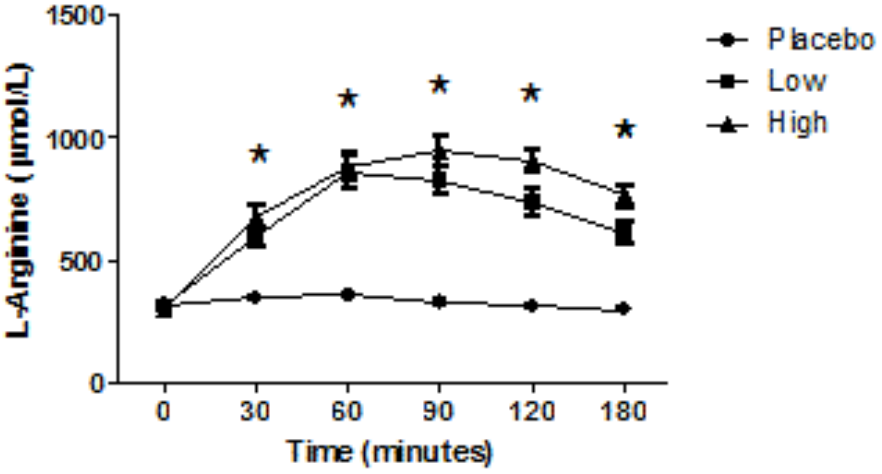

Figure 7. Mean ± SEM plasma L-arginine concentrations over time by condition. *=indicate a significant difference between the L-arginine conditions and the placebo condition.

APPENDIX E

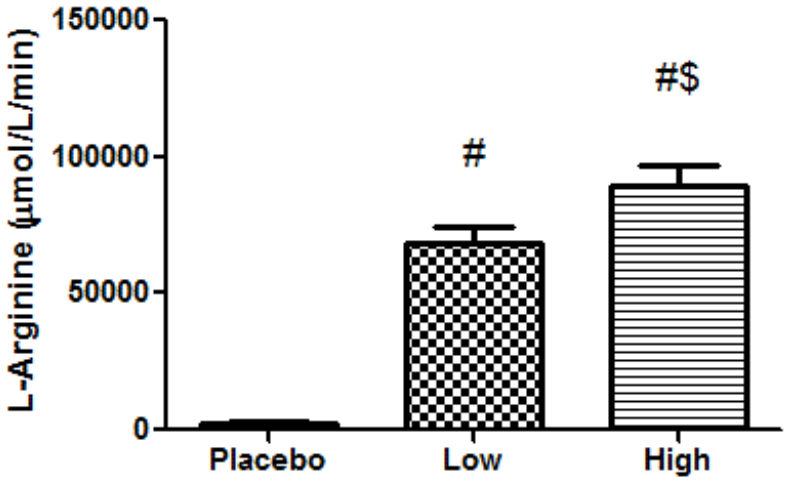

Figure 8. Integrated areas under the curve L-arginine concentrations following the two treatment conditions and control condition. #=indicates a significant difference from the placebo condition. $=indicates a significant difference between from the low condition.

APPENDIX F

A Graduate Student Pilot Project Submitted to the Faculty of Physical Education and Recreation
Ethics Committee

Title: ***The effects of a low and high dose of L-arginine supplementation on physiological responses at rest.***

What (exactly) are you doing?

L-arginine is considered to be a conditional or semi-essential amino acid in our body. It can be synthesized in the kidney (most) and liver (least), however, under conditions of various types of stress, including exercise, L-arginine may become essential and supplementation from an external (dietary) source is required. Thus, it can be consumed in the diet from a variety of sources (nuts, seeds, grains, dairy products, meat) or from a powder or capsule commercially available through various nutrition stores (McConell, 2007). L-arginine has been associated with a number of metabolic and hormonal responses in the body that can be linked to exercise and performance, but the physiological mechanism(s) by which L-arginine supplementation may influence acute and chronic exercise performance are not fully understood. For example, L-arginine has been associated with the detoxification of ammonia formed during nitrogen catabolism of amino acids via the formation of urea (Schafer et al. 2002). In addition, L-arginine is utilized as a precursor of a variety of biologically active compounds such as nitric oxide and creatine and has been shown to promote an increase in anabolic hormones such as human growth hormone and glucagon (Dela et al. 1990; Hendler et al. 2001). The

majority of this latter research demonstrated an ergogenic effect in certain clinical populations using a variety of L-arginine dosages; but research examining the ergogenic potential and underlying physiological reasons for these changes that may contribute to an improvement in sport performance and exercise training remain unclear (Ceremuzynski et al., 1997; Rector et al., 1996; Cheng & Baldwin, 2001; Nagaya et al., 2001; Stevens et al., 2000; Campbell et al., 2006; Santos et al., 2002; Buford and Koch, 2004; Elam et al., 1989; Walberg-Rankin et al., 1994; Able et al. 2005). Interestingly, several of these latter studies and a bulletin released by the Mayo Clinic (www.mayoclinic.com) state that there are "no established standardization for oral arginine products". Currently, there are no published studies that have determined an effective relative dose of L-arginine on any particular physiological response. Studies examining L-arginine typically give an absolute dose varying from 0.5 grams to 35 grams per day given either intravenously or orally. However, oral consumption of L-arginine is most practical and logical in the non-clinical setting. Therefore, we are proposing to examine different dosages of L-arginine supplementation using a within subject, randomized, double blind, placebo controlled trial. This research is necessary to establish what amount of L-arginine will provide a targeted physiological response that can subsequently be used in future investigations of the effectiveness of L-arginine on exercise and sport performance.

Why (what benefits are there to the participants, to society, or to further research? What are you trying to find out?)

The purpose of this study will be to examine the effectiveness of two doses of L-arginine on hormonal (ghrelin and growth hormone-releasing hormone) responses in the blood. This project will provide valuable information about how much L-arginine is required to be ingested to elicit a particular physiological response. One benefit to participation in this study would be that all subjects will be provided with their personal research results if desired to personally learn more about this particular supplement. It is hoped that this research will reveal certain dose-response characteristics that will also aid society and the scientific community that would be consuming or prescribing L-arginine as a supplement.

Who are the Participants?

A sample of 15 healthy male subjects (based on a statistical power calculation) between the ages of 18 and 39 will be recruited through word of mouth from the University of Alberta. We are only testing males as some of the hormonal responses may very based on the menstrual cycle that is difficult to control for as well as only limited funding has been obtained for this research study at this time that limits the number of participants we can include. Based on previous research that has examined some of the primary outcome measures we will be measuring from L-arginine supplementation (ghrelin, growth hormone-releasing hormone) but from a different population (Dela et al. 1990; Hendler et al. 2001), an effect size was calculated to be between 0.83 – 1.30 [($\times^1$ - $\times^2$) / SD, Cohen, 1977; Thomas and Nelson, 2005]. Thus, a sample size of 12 participants will provide us with a statistical power > 0.80 (Cohen, 1977). However, we will recruit 15

subjects in case of attrition or other experimental issues. Subject selection criteria will include being free from disease, food allergies of any kind or any other condition that would prevent participation in this type of nutritional supplement study. This will be conducted by verbal interview with each subject.

Where will the study take place?

The testing will be performed in the exercise physiology lab (P340-344). Biochemical analyses will be performed in the exercise biochemistry lab (E443). These labs are within the Faculty of Physical Education and Recreation and the supervising professor has a current authorization to work with human blood in each of these laboratories (E.H. & S., U. of A. Permit #A19).

How are you going to do it?

Individuals will be contacted by word of mouth and will be asked to attend an information and orientation meeting. At this meeting, participants will be informed of the purpose and procedure of the study and screened for inclusion criteria verbally and be instructed on how to complete a 1-day dietary record. Once the participant has agreed to participate and completed all forms they will be scheduled for the testing. As well, the subjects will be given a tour of the lab and shown the equipment to be used for the study.

The testing will involve 4 separate visits to the lab: the first is for the orientation meeting. The next three visits will be the 2 dosages and the 1 placebo control

condition in random order when blood sampling will occur before and after the consumption of L-arginine in capsule form taken with water. A registered nurse will take all the blood samples.

Each subject will be given a dietary intake form that has space for recording the type and amount of all the food and beverages (including water) consumed over one day. They will be instructed on how to be accurate in recording this information. This record will be analyzed with a software program (Food Processor II) that will determine the nutritional makeup of the subject's diet. The food record will be modified to achieve a balance of each subject to obtain a 0.80 $g \cdot kg^{-1} \cdot day^{-1}$ of mixed food protein (so that similar L-arginine are consumed in the diet the day before the experiment) at the same time as maintaining the total calories equal to the subject's original diet record. This form will then be returned to each subject and they will be required to follow this same diet the day before each of the 3 experimental sessions.

We will record age, measure height, body mass and have the participants complete a Godin Leisure Time Questionnaire (Godin, 1985) to assess physical activity levels.

Hormone response to the various dosages of L-arginine

The participants will arrive on different days to perform the trials separated by at least 5 days and will be asked not to exercise (day off) 24 hours prior to the

morning of each experiment. The subjects will arrive first thing in the morning (between 07:00 and 08:00 am) in the lab after an overnight fast (10 hour fast). Upon arrival, their body mass will be measured and then a registered nurse will then insert a 22 gauge cathelon, secure it with tape and obtain a fasted blood sample (10 ml) with a syringe. Two ml's of sterile saline solution will be used to keep the cathelon clear and open during each experiment. Following this, each subject will be provided one of 3, randomized treatment conditions: 1. Placebo-control: (capsules with a mixture of whole wheat flour take with water), 2. Low dose of L-arginine in capsule form: 0.075 $g \cdot kg^{-1}$ or 3. High dose: 0.15 $g \cdot kg^{-1}$ of L-arginine capsules. These doses encompass the range reported in the literature. Note that L-arginine is commercially available in Canada and can be purchased anywhere food or nutritional supplements are sold. An individual not involved in the research study in any way will be recruited to do the randomization and code the drinks so that the subject and researchers are blind to the experimental condition. Blood will be drawn at rest (fasted), 30, 60, 90, 120 and 180 minutes post consumption. Additionally, after the 90 minute and last blood sample (180 min) the subjects will be asked to provide a urine sample in a sterilized cup designed for this type of collection. Subjects will be required to remain in the laboratory for the entire duration of the testing protocol but can read, do homework or use a computer. L-arginine concentration in the serum blood will be measured using high performance liquid chromatography (HPLC) or by a spectrophotometric method. Ghrelin and growth hormone-releasing hormone will be analyzed in blood using commercially available assays. The total number of

blood samples is 6 and this amounts to a total of 60 ml's for each experimental session which represents no risk to the subject.

Blood Analysis

All the blood assays are routinely performed in the laboratory.

Statistical Analysis

The means and SEM will be determined for all dependent variables. Each variable will be initially analyzed using a 3 treatment (placebo and 2 doses of L-Arg) by 6 times (blood samples) factorial ANOVA with repeated measures on both levels. A multiple comparison procedure will be used to determine any further differences where any significant F-ratios appear. Alpha will be set a priori at $p<0.05$.

How long will it take?

Orientation meeting =	30 minutes
Completing diet record at their home =	Total of 30 minutes throughout the day.
3-Experimental trials =	3*3.5 hours = 10.5 hours
Total testing time =	11.5 hours.

Testing time does not include travel to and from the lab.

What are the qualifications of the research personnel?

All testing will de done under supervision of the supervising professor. Any assistants that may be involved will be graduate students in exercise physiology. A registered nurse will be used for the blood taking procedures.

What are the potential risks of involvement in the study (worst case scenario and likelihood of occurrence) – both to the participants and the researcher?

There is little to no risk to the researchers or research assistants other than general hazards common to working in a laboratory setting. The blood samples are performed under sterile conditions but there is a risk of infection at the site of the blood sample if not properly cared for. This risk will be minimized through sterile procedures, cleanliness and the use of a band-aid. Universal procedures will be followed for all testing procedures, that is, rubber gloves, lab clothing and cleaning all areas with 10% bleach solution that may be in contact with biohazardous material as outlined by the U. of A. Environmental Health and Safety guidelines. The principal investigator has a permit with this office to work with the biohazardous materials that will be encountered in this study (permit #A19).

What procedures are in place to deal with potential risks, or what steps have been taken to minimize the possible risks?

All personnel are required to have completed the WHMIS – Biosafety course and have completed a Hepatitis B inoculation. This ensures that all personnel are versed in proper procedures for dealing with biohazardous materials. Only

personnel trained in phlebotomy procedures will be allowed to take blood samples from the subjects. The laboratory environment will be maintained in a safe manner and all personnel will follow emergency procedures common to the lab areas.

References available on request.

APPENDIX G

Table 1. Ghrelin concentrations overtime following both treatments and control condition

Time (min)	Low	High	Placebo
0	121.23 ± 14.93	112.29 ± 15.92	97.95 ± 13.7
30	103.85 ± 16.11	85.92 ± 11.56	100.36 14.15
60	101.13 ± 17.26	95.17 ± 13.07	104.31 ± 14.89
90	109.64 ± 15.55	98.47 ± 13.39	106.85 ± 14
120	110.63 ± 12.09	104.71 ± 16.26	114.90 ± 17.23
180	122.21 ± 16.73	107.37 ± 14.8	118.74 ± 17.15

*Values are means ± SEM and expressed in pg/mL, $p<0.05$.

APPENDIX H

Table 2. GHRH concentrations overtime following both treatments and control condition

Time (min)	Low	High	Placebo
0	123.50 ± 52.14	135.73 ± 50.91	117.27 ± 43.3
30	119.36 ± 44.52	100.85 ± 40.95	131.65 ± 38.15
60	103.78 ± 37.47	131.82 ± 58.6	117.09 ± 45.3
90	116.11 ± 44.19	112.75 ± 43.86	107.51 ± 39.67
120	117.47 ± 46.86	111.01 ± 45.57	136.34 ± 47.55
180	114.60 ± 47.29	122.82 ± 45.08	112.34 ± 37.95

*Values are means ± SEM and expressed as pg/mL, $p<0.05$.

APPENDIX I

Table 3. GH concentrations overtime following both treatments and control condition

Time (min)	Low	High	Placebo
0	0.83 ± 0.23	0.98 ± 0.33	1.28 ± 0.45
30	1.78 ± 0.85	1.3 ± 0.63	2.06 ± 0.83
60	1.87 ± 1.87	1.08 ± 0.43	1.36 ± 0.49
90	1.77 ± 0.89	1.26 ± 0.42	0.89 ± 0.21
120	1.5 ± 0.43	0.85 ± 0.18	0.71 ± 0.11
180	0.79 ± 0.11	1.41 ± 0.55	1.06 ± 0.29

*Values are means ± SEM and expressed in μg/L, $p<0.05$.

APPENDIX J

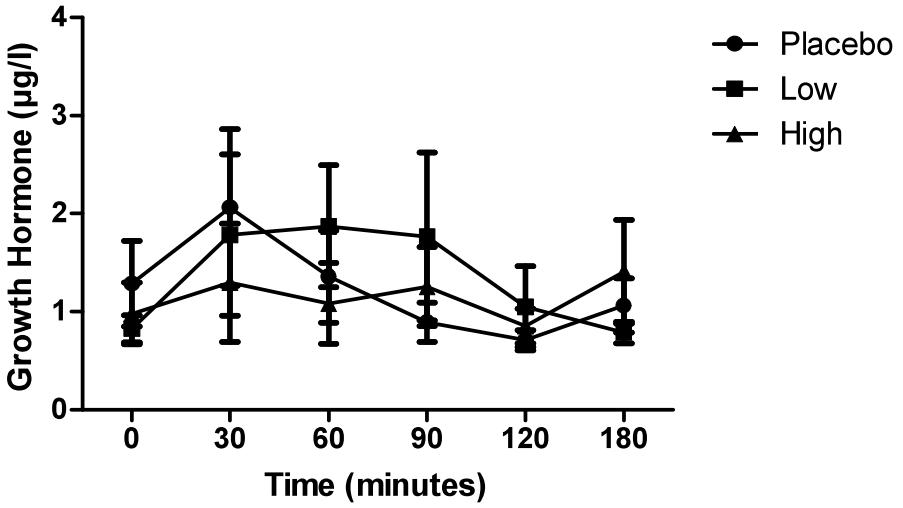

Figure 9. GH concentrations over time following the three treatment conditions Values expressed as mean ± SEM, $p<0.05$.

APPENDIX K

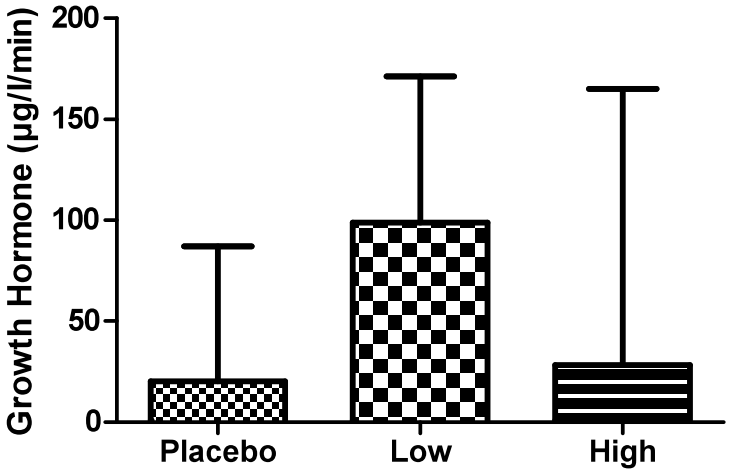

Figure 10. Area under the curve values for GH following the three treatments. Values expressed as mean ± SEM, $p<0.05$.

APPENDIX L

Growth Hormone Assay General Principle: This Enzyme Immunometric Assay (EIA) follows a typical one-step capture or 'sandwich' type assay. The assay makes use of two highly specific monoclonal antibodies: A monoclonal antibody specific for hGH is immobilized onto the microwell plate and another monoclonal antibody specific for a different region of hGH is conjugated to horse radish peroxidase (HRP).

hGH from the sample and standards are allowed to bind simultaneously to the plate and the HRP conjugate. The washing and decanting steps remove any unbound HRP conjugate. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution.

The absorbance is measured on a microtiter plate reader at 450nm. The intensity of the colour formed by the enzymatic reaction is directly proportional to the concentration of hGH in the sample. A set of standards is used to plot a standard curve from which the amount of hGH in subject samples and controls can be directly read.

L-arginine Assay General Principle: Arginine is reductively condensed with pyruvate to octopine by the action of the enzyme octopine dehydrogenase, ODH. This specific reaction is the basis of the measurement of arginine.

The decrease in NADH concentration, measured by the change in absorbance at 339nm is proportional to the amount of arginine (light path of

10mm).

Optimized conditions for measurement: the equilibrium of the reaction is in favour of octopine production, especially at neutral pH. The measurements are therefore carried out at pH 7.0. In addition, the second substrate of the reaction, pyruvate, is used in a high concentration.