THE RELATIONSHIP OF TESTOSTERONE, APPETITE, FOOD INTAKE AND EXERCISE IN MALE ADOLESCENTS

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Nutritional Sciences
University of Toronto

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2019

ABSTRACT

The hypothesis that testosterone has an interactive relationship with food intake and high intensity exercise in adolescent males was explored in three experiments. Combined (Experiment 1) and separate (Experiment 2) effects of acute glucose and protein ingestion on testosterone were observed. In Experiment 1, testosterone decreased by $18.6 \pm 3.1\%$ ($p < 0.01$) after one hour of ingesting the combined glucose/protein beverage. In Experiment 2, testosterone decreased acutely 20 min after both protein and glucose ingestion with the decrease continuing after protein but not glucose after 65 minutes ($p = 0.0382$). No associations between testosterone and appetite or food intake were found. Experiment 3 aimed to define the effect of sustained bouts of high intensity exercise on testosterone. The effect of high intensity aerobic exercise (HIEX) on testosterone in adolescent males was measured through two sessions of either: 1) three 10-minute bouts of HIEX cycling at 75% VO$_2$peak or 2) rest. Plasma testosterone concentrations increased by $21.5 \pm 13.6\%$ after three 10-minute bouts of HIEX when compared to rest ($p = 0.0215$) but testosterone was not correlated with appetite or appetite related hormones. Thus, the results of this research show the novel effects of acute nutrient intake and high intensity aerobic exercise on testosterone levels in adolescent males but no relationship with appetite and food intake regulation was established. Therefore, this research does not support the hypothesis that testosterone has a short-term interactive relationship with food intake.
ACKNOWLEDGMENTS

I dedicate this thesis to my late parents who sacrificed their lives and left our birthplace and home to seek a better future for my brother and I. I am forever grateful for the unconditional love of both of them. My mother never lived to see me grow into the person I am today but would be proud. My father, in spite of losing his own battle with cancer during the preparation of this dissertation, has shown me strength, support and love throughout my graduate studies and made me appreciate life more every day. Loss would never be painful if weren’t for love and happiness.

I also dedicate this to my life partner Dr. Shannon Vettor who has been there for me during the most difficult time in my life and given me strength when I felt my weakest. I feel fortunate every day to be waking up and knowing your love, intelligence, support and compassion can get me through anything. I’m excited to raise our soon to be born son Simon with you and begin this new chapter in our lives together.
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<tr>
<td>α-MSH</td>
<td>α- Melanocyte Stimulating Hormone</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>5α-Dihydrotestosterone</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-Related Peptide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ARC</td>
<td>Arcuate Nucleus</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>CART</td>
<td>Cocaine-and-Amphetamine-Regulated-Transcript</td>
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<tr>
<td>CLIA</td>
<td>Chemiluminescent Assay</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DMH</td>
<td>Dorsomedial Hypothalamus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>FFM</td>
<td>Fat-Free Mass</td>
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<tr>
<td>FI</td>
<td>Food Intake</td>
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<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
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<tr>
<td>GCMS</td>
<td>Gas Chromatography Mass Spectrometry</td>
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<td>GLP-1</td>
<td>Glucagon-Like-Peptide-1</td>
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<td>GnRH</td>
<td>Gonadotropin-Releasing Hormone</td>
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<td>Abbreviation</td>
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<tr>
<td>HPGA</td>
<td>Hypothalamic-Pituitary-Gonadal Axis</td>
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<td>IA</td>
<td>Immunoassay</td>
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<td>IGF-1</td>
<td>Insulin-Like Growth Factor-1</td>
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<tr>
<td>Kcal</td>
<td>Kilocalories</td>
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<tr>
<td>LatH</td>
<td>Lateral Hypothalamus</td>
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<tr>
<td>LCMS</td>
<td>Liquid Chromatography Mass Spectrometry</td>
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<tr>
<td>LDL</td>
<td>Lipoprotein Cholesterol</td>
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<td>LH</td>
<td>Luteinizing Hormone</td>
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<tr>
<td>LHCGR</td>
<td>Luteinizing Hormone/Chorionic Gonadotropic Receptor</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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<tr>
<td>POMC</td>
<td>Pro-Opiomelanocortin</td>
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<tr>
<td>PYY</td>
<td>Peptide Tyrosine Tyrosine</td>
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<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SHBG</td>
<td>Sex Hormone Binding Globulin</td>
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<td>VAS</td>
<td>Visual Analogue Scale</td>
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<td>VMH</td>
<td>Ventromedial Hypothalamus</td>
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List of Publications

Peer Reviewed Articles:

**Chapter 4**  
**Schwartz, A.,** Anderson, G.H., Patel, B.P., Vien, S., McCrindle, B.W., Hamilton, J. Acute decrease in serum testosterone after a mixed glucose and protein beverage in obese peripubertal boys. Clinical Endocrinology 2015; 83(3); 332-338.

**Chapter 5**  

Publications Related to the Thesis:


Hunschede, S., **Schwartz, A.,** Kubant, R., Thomas, S.G., Anderson, G.H. The role of IL-6 in exercise-induced anorexia in normal-weight boys. Applied Physiology, Nutrition and Metabolism 2018; 43; 979-987


Kucab, M., Boateng, T., Brett, N.R., **Schwartz, A.,** Totosy de Zepetnek, J.O., Bellissimo, N. Effects of eggs and egg components on cognitive performance, glycemic response, and subjective appetite in children aged 9-14 years. Current Developments in Nutrition. 2019; 3(S1); P14-017-19.

Published Abstracts:


Hunschede, S., Schwartz, A., Kubant, R., Akilen, R., Thomas, S., Anderson, G.H. Mechanisms of Appetite Suppression After High Intensity Exercise in Lean and Obese Boys. The FASEB Journal (2016); 30; S1


Chapter 1

INTRODUCTION
1 INTRODUCTION

During adolescent pubertal development, obtaining the necessary nutrients required for healthy growth into adulthood is critical. Though most Canadian adolescents meet their daily nutritional recommendations and ingest acceptable portions, 30% of Canadian children and adolescents are overweight or obese [1]. Vast epidemiological evidence suggests that excess adiposity in adolescence persists into adulthood; excess BMI and obesity during adolescence are connected to mortality [2].

There is contradictory evidence in the literature with regards to excess adiposity and the onset of male puberty. A Danish study indicated that lower vocal timbre was reached earlier in obese adolescent males than normal-weight ones [3]. However, other population studies have demonstrated that the ‘relative risk’ to stay in pre-pubertal status in males as defined by pubertal staging is higher in those with a higher body mass index (BMI) [4]. Further studies following monozygotic and dizygotic twin pairs from birth using peak growth velocity and peak height concluded that growth during puberty is strictly regulated by genetics [5]. These conflicting findings highlight the difficulty of measuring when puberty begins in males due to inconsistencies in defining the parameters of pubertal onset.

Given the known inverse relationship of testosterone and excess body fat in adult males, focusing on testosterone and obesity/growth in this population may be beneficial, [6, 7]. Testosterone is a steroid hormone that increases during onset of male puberty and shows a strong correlation to growth and velocity during male puberty [8]. A recent paper demonstrated that obesity and lower testosterone in adolescent males led to comorbidities in adulthood such as insulin resistance [9].

Considering the importance of testosterone in adolescent male development and the intimate relationship between obesity and food intake, there is a need for understanding how food intake modifies testosterone levels in teenage males, as this interaction could play a role in male development. There is scarcity of well-controlled research examining the acute effect of food intake on testosterone, and none to date that examines the effect of foods on testosterone in adolescent males. In adults, a decrease of postprandial testosterone was demonstrated following an oral glucose tolerance test (OGTT) challenge [10], but there are no studies utilizing protein in a similar manner to glucose and no studies exist to determine the effects of food intake on testosterone in adolescent males.
In contrast to the effect of food intake on acute testosterone levels, there is compelling evidence to show that testosterone levels increase after both acute bouts and habitual engagement of physical activity in adult males [11-13]. However, limited evidence exists on how these levels are affected in the adolescent population due to a myriad of methodological issues such as the need to reach an intensity threshold in these activities in order to stimulate peripheral testosterone production [14]. Teenagers in Canada consistently fall short of obtaining the minimal requirement of physical activity. Approximately 45% of boys aged 12-17 years fail to meet the intensity levels necessary for physiological benefits [15]. Higher intensity levels of objectively measured physical activity have been shown to be inversely related to adiposity in adolescents; those who engaged in at least 60 minutes of moderate-to-vigorous physical activity per day were leaner in comparison to those who did not [16].

In view of the suspected interplay between testosterone, nutrition and physical activity in teenage males, it is necessary to have research defining how they may interact to increase our understanding of how nutrition and exercise affect development. Therefore, this dissertation will investigate the effects of nutrients and exercise on testosterone and examine the relationship testosterone has with appetite regulation in adolescent males.
Chapter 2

REVIEW OF LITERATURE
2 REVIEW OF LITERATURE

2.1 Introduction

The purpose of the review is to provide rationale for the research presented in this dissertation. This review begins by addressing the prevalence and issues surrounding adolescent obesity. The following section reviews pubertal development in males since this is the specific population studied throughout this thesis. Hormonal regulation during puberty will be discussed and, furthermore, specific pubertal abnormalities pertinent to this research will be addressed. Section four deals specifically with hormonal regulation of appetite and food intake and section five synthesizes the previous two sections by providing insight into appetite during puberty. Section six discusses the relationship of physical activity/exercise and testosterone and finally, a summary and research rationale will be provided in section seven.

2.2 Overweight and Obesity in Adolescence

Obesity is a medical condition where excess body fat leads to negative health consequences and reduced lifespan. It affects 1.9 billion adults [17] and approximately 10% of children worldwide [18]. It is defined by body mass index (BMI), which is calculated by weight divided by the square of the height. This measure has been shown to be a reasonable estimate of adiposity in the general population, though begins to break down in active individuals with higher levels of lean body mass [19]. In adults, the World Health organization defines a BMI of >25kg/m$^2$ as overweight and a BMI of >30kg/m$^2$ as obese [20].

In children and adolescence, healthy weight and height varies with age and sex and is defined using BMI percentile. For boys 2-20 years of age, a BMI percentile of 85$^{th}$ to less than the 95$^{th}$ percentile is defined as overweight, whereas anything above the 95$^{th}$ percentile is considered to be obese [21]. In Canada it is estimated that nearly a third of 5 to 17-year olds are overweight and 19.5% of boys are obese [22, 23]. Childhood and adolescent obesity often persists into adulthood and is associated with chronic illnesses such as hypertension, hyperlipidemia, cardiovascular disease, diabetes and fatty liver disease in addition to premature mortality [24, 25].
In addition to metabolic disorders, excess adiposity has an impact on growth. Though adolescent males with overweight or obesity show advanced bone age in comparison to normal weight boys, their bone mineral content is still lower than what would be expected for their body weight [26]. Furthermore, excess adiposity in boys with obesity is associated with a delay in pubertal development [27], though paradoxically, the opposite holds true for females [28]. This pubertal delay may be a consequence of decreased testosterone levels in obese adolescent boys when compared to lean adolescents [29, 30]. The differences of testosterone between boys who are obese vs. lean persists throughout puberty into adulthood [9] and may result from diminished testicular function due to excess adiposity [31]. Understanding how excess body fat can affect normal pubertal development in males during a critical period in male growth is an important and often overlooked facet of obesity prevention.

2.3 Puberty and Growth in Males and Normal Development

*Pubertal Onset*

The onset of ‘true’ male puberty begins with the activation of the hypothalamic-pituitary-gonadal axis (HPGA), when gonadotropin-releasing hormone (GnRH) pulsatility increases after a quiescent period and stimulates the production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland [32, 33] (Figure 2.1). These gonadotropins signal spermatogenesis (FSH) and the production of testosterone (LH) in the testes, resulting in secondary sexual characteristics associated with puberty in boys. Though normal puberty is said to begin between nine and 14 years of age in boys [34], average age of pubertal onset in males is difficult to define and varies as a result of a multitude of factors including ethnicity, genetics, and nutritional status.
Figure 2.1- Onset of normal puberty in boys. Puberty begins with activation of the hypothalamic-pituitary-gonadal axis (HPGA) stimulating gonadotropins (FSH & LH) to bind to target cells in the testicles resulting in spermatogenesis and development on secondary sexual characteristics.

Though physiological signals from the HPGA indicate that puberty has initiated, the exact mechanism responsible for pubertal onset remains to be elucidated. Puberty is dependent on an interplay between genetic and environmental factors, and various studies indicate that in well-nourished populations, genetics influences approximately 40-80% of the variation in the timing of puberty in boys [35-38]. The summation of research for the genetic influence on pubertal timing has been drawn primarily from single-gene disorders and indicates that though there are many genes involved, the gene which encodes the kisspeptin protein (KISS1) as well as its corresponding G-protein coupled receptor 54 (GPR54) and gonadotropin-releasing hormone receptor (GnRH) play pivotal roles in pubertal timing. KISS1/GPR54 signaling acts in the initiation of GnRH secretion at puberty by acting on the GnRH neuron which expresses GPR54, resulting in the release of gonadotropins[39, 40]. Evidence from animal models provide support in that KISS1/GPR54 mRNA levels in primates increase during puberty [41] and additionally, mice with GPR54 and KISS1 knockouts fail to enter puberty [42, 43].
Given that genetics do not explain all of the variation seen with pubertal timing, environmental influences, therefore, must also play an important role in pubertal onset and potentially mediating the genetic regulation. One of the strongest and most influential factors from the environment may be nutrition. The association of overall diet quality with the timing of puberty in both boys and girls has only been analyzed in the DONALD study [44]. Using dietary records and a nutritional quality index, a multivariate analysis revealed that, after adjustment for sex, maternal overweight, energy intake at baseline, and respective anthropometry at baseline, a lower nutritional quality index in prepuberty was still associated with an earlier age of pubertal onset; children with a lower diet quality according to their nutritional quality index score entered puberty ~0.4 y earlier than children with a higher diet quality. Though there are several limitations with using dietary records, it does demonstrate that diet can have an effect on pubertal timing. This is further evidenced when looking at protein intake and protein quality. When compared to animal protein, a higher vegetable protein intake at 5-6 years of age has been shown to delay puberty in both males and females [45]. This is not surprising given that animal protein, in particular dairy, stimulates secretion of insulin thereby decreasing IGF binding protein 1 and increasing availability of IGF-1 [46], the significance of which with puberty is discussed further in section 2.5.2. Energy imbalance (caloric surplus) resulting in obesity is an additional important nutritional consideration and the relationship between obesity and puberty will be discussed in depth in section 2.5.4.

A variety of other physical and psychosocial stressors may also influence the timing of puberty. Physical stressors such as high levels of exercise have been demonstrated to attenuate HPGA activity in adult male runners [47, 48], but whether this translates to pre-pubertal males remains unknown. Psychosocial factors such as parental absence, income status and ethnicity have also been shown to affect entry into puberty for girls, but there is a gap in evidence for similar findings in males [49, 50]. Some evidence shows similar trends in boys entering puberty at different rates based on race/ethnicity, but the socioeconomic and environmental factors surrounding different ethnicities remain to be addressed [51].

**Pubertal Characteristics and Development**

Measurement of puberty using the Tanner Stages is discussed in detail in section 2.3.3.1. The first manifestation of puberty in boys is typically testicular enlargement (gonadarche).
Testicular volume, correlated with Tanner Stage, is one of the critical measures for pubertal development of boys in the Tanner Scale and ranges between ≤ 3ml (stage I) to ≥ 20ml (stage V). A testicular volume of > 3.0ml indicates entry into puberty and is an important element of genital Tanner Stage 2 [52]. After approximately one year of testicular enlargement, the size of the penis gradually begins increasing and developing adult proportions [53], and this coincides with the development of varying levels of pubic hair around the genitals, torso, legs, face and axillary regions. Pubic hair development (pubarche) results from increased production of adrenal and gonadal dehydroepiandrosterone (DHEA) in males which converts to dihydrotestosterone in hair follicles and dermal exocrine glands stimulating hair growth [54, 55]. Additionally, the increased levels of DHEA play a major role in adolescent acne [56, 57].

The increased levels of androgens in males affect other organs such as the larynx, muscles, and bones. Abrupt changes in voice characteristics (deepening) have been noted to occur between Tanner Stage 4 and 5 [58]. Under the influence of testosterone, males have significant increases of bone and muscle size and strength concomitant with decreased adiposity; 50% of adult body weight is gained during puberty and peak weight velocity (9kg/y) occurs at the same time as peak height velocity, which ceases with epiphyseal closure at approximately 17 years of age [59]. The bone growth of males is reflected by temporary increases seen in the levels of alkaline phosphatase (corresponding to osteoblast activity) due to the rapid bone growth which peaks at approximately Tanner Stage 3 [60, 61]. In addition to increased bone mass, adult males have approximately 150% of the lean body mass of females and nearly twice the number of myocytes [62].

Though puberty is associated with the ‘classical’ physiological changes mentioned above, other important systems also go through modifications during adolescence. Hemoglobin concentrations increase in adult males more so than in females as a result of increased erythropoietin/red blood cell production from elevated testosterone production [63]. Total cholesterol also increases, peaking during early puberty along with triglycerides and blood pressure. However, low-density lipoprotein cholesterol (LDL) peaks during late puberty and high-density lipoprotein cholesterol concentration remain somewhat constant, though there may be a small decrease from early to mid-puberty [64].

Sequentially, male puberty usually begins with gonadarche [52] followed by the pubarche. Though some ethnicities may not show pubarche this early, Tanner stage 3 pubarche occurs
approximately 1 to 1.5 years after initial testicular enlargement [59]. A pubertal growth spurt occurs at approximately genital stage 3 and 4 at the same time as sperm development (spermarche) [65]. Masculinization such as facial hair appearance and larynx enlargement occurs at around genital stage 4 and male puberty is typically completed or near completion by 16-18 years of age.

### 2.3.1 Sex Hormones

#### 2.3.1.1 Physiology of Luteinizing Hormone and Role in Male Puberty

Luteinizing hormone (LH) is a gonadotropin and glycoprotein which is produced within the gonadotropic cells of the anterior pituitary gland in the hypothalamus. The hypothalamus releases GnRH, which controls the release of LH [33]. Throughout fetal development, fluctuations in circulating LH can be observed with peak values occurring before 20 weeks and decline again before birth [66-69]. Immediately after birth, neonates experience a surge in LH due to the withdrawal of maternal derived estrogen, and this surge is larger in male than female infants [70]. In male infants, LH peaks at approximately 1 to 3 months of age, declining rapidly and reaching a nadir at 4-9 months [71, 72]. The steroidogenic actions of LH are exerted primarily through cAMP-mediated events in the gonads via the enzymes of the Leydig cells [73, 74]. LH controls both the amount of cholesterol entering the Leydig cell and the production of testosterone from cholesterol within the cell itself [75].

The GnRH pulse generator begins to mature approximately 1 to 2 years prior to the onset of puberty and testosterone production, which leads to a gradual increase in frequency and amplitude of LH pulsatile secretion that occurs nocturnally [76, 77]. As puberty progresses, these secretions occur with increased frequency throughout the day and as part of the hypothalamic-pituitary-gonadal axis (HPGA) [78], and in turn stimulate steroidogenesis in the testes (see 2.3.1.3) [79]. In pubertal boys, daytime plasma LH values were above 0.3 IU/L, with periods of values of 0.1-0.3 IU/L in addition to short periods of undetectable levels. Nocturnally, up to 4.7 IU/L were found in all boys with a higher frequency of pulses at night [80].
2.3.1.2 Physiology of Follicle Stimulating Hormone and Role in Male Puberty

Follicle-stimulating hormone (FSH), a gonadotropin and glycoprotein similar to LH, is produced within the gonadotrophic cells of the anterior pituitary gland in the hypothalamus. The hypothalamus releases GnRH which controls the release of FSH [33]. The primary function of FSH is gamete production during the fertile phase of life [81], and in males this is manifested through spermatogenesis in the Sertoli cells of the gonads [82]. Mutations in the FSH receptor have resulted in delayed sexual maturity and diminished testosterone production and reductions in fertility [83-85]. In addition to fertility, FSH plays a role in thyroid regulation [86]. FSH is important to normal pubertal development; deficiency of FSH or the loss FSH receptor signaling decreases the number of Sertoli cells and diminishes testicular size [87] in addition to reduced adult body size [88] and delayed puberty due to hypogonadism [89].

2.3.1.3 Physiology of Testosterone and Role in Male Puberty

Testosterone is a steroid hormone and plays a pivotal role in male pubertal development. Production of testosterone is initiated by secretion of LH from the pituitary gland, which binds to the G protein-coupled LH/chorionic gonadotropic receptor (LHCGR) on the surface of the Leydig cells situated on the testes [90]. The activation of the LHCGR stimulates intracellular concentrations of cAMP in the Leydig cell resulting in the production of proteins necessary for steroidgenesis. This activation subsequently leads to the conversion of intracellular cholesterol into testosterone [74]. LH acts as a central regulatory factor within the Leydig cells as it regulates both the amount of cholesterol entering the cell and the production from cholesterol within the cell itself [75].

Cholesterol is incorporated into the cell from either receptor mediated endocytosis via LDL’s or synthesized de-novo starting from acetyl CO-A and stored in cytoplasmic lipid droplets [75]. Testosterone synthesis requires conversion of cholesterol into testosterone through five different enzymatic steps which can take approximately 30 minutes [91] (Figure 2.2). The cholesterol molecule’s side chains are shortened through C22 and C20 hydroxylases (Cytochrome P450Scc) followed by cleavage of the bond between C20 and C22, leading to production of pregnenolone within the mitochondria of the Leydig cell [92, 93].
Once pregnenolone is formed, it enters the endoplasmic reticulum of the Leydig cell and follows either the Δ4 or Δ5 pathway depending on the location of the double bond in the pregnenolone steroid. The Δ4 pathway involves dehydration and conversion to progesterone and then into 17-hydroxy-progesterone whereas the Δ5 pathway is more common in humans [94] and is converted to the intermediate 17-hydroxy-pregnenolone which in turn is converted into dehydroepiandrosterone (DHEA) [95]. Both 17-hydroxy-progesterone from the Δ4 pathway and DHEA from the Δ5 pathway are used to produce the intermediate androstenedione, which is then converted to testosterone [96].

During transport, testosterone is mainly bound to albumin or to sex hormone binding globulin (SHBG) which is produced by hepatocytes. In normal men, only 2% of total testosterone circulates freely (unbound), while 44% and 54% are bound to the binding proteins SHBG and albumin, respectively [97, 98]. Though the binding affinity of testosterone to SHBG is much higher (approximately 100 times greater), the greater concentration of albumin results in a fairly close binding capacity between the two proteins [99]. It is important to note that the movement of testosterone from the blood into cellular compartments such as the brain or muscle is reduced by SHBG but not by albumin [100, 101]. Thus, unlike SHBG, albumin allows for biological action of testosterone with the intra-cellular nuclear androgen receptor (bioavailability) [99]. Since binding proteins cannot move across cellular membranes, testosterone must enter target cells in the following ways, though the most relevant pathway of these remains to be elucidated:

i) **Diffusion- disassociation**

Diffusion- disassociation of testosterone from binding proteins occurs primarily in the capillaries near target cells as a product of the interaction of endothelial glycocalyx resulting in structural modifications that allow testosterone to be set free from the extracellular space into the intracellular space [102]. From here, testosterone can enter the cell freely across the membrane through diffusion. It then binds to an intracellular receptor and enters the nucleus to regulate gene expression [103, 104].

ii) **Receptor-mediated endocytosis of steroid**

While still bound to SHBG, testosterone binds to a cell importer protein called megalin [104, 105]. The entire hormone carrier is degraded within the cell, and the ligand testosterone
hormone is released into the free cytoplasm, where testosterone is broken apart from the SHBG and can enter the nucleus [106].

**iii) Non-genomic mechanisms of testosterone action**

The established model of genomic testosterone transport and transcription dictates that testosterone, like other steroid hormones, freely crosses the plasma membrane of the cell and binds to specific receptor proteins within the cytoplasm. The bound steroid receptors bind to homodimers and heterodimers in target gene promoters and activates/de-activates transcription leading to protein synthesis [107-111]. This process may take up to several hours after steroid exposure and takes additional time for messenger RNA to be translated into proteins which then illicit signaled responses [112]. Steroid hormones can indeed affect cellular processes in a non-genomic fashion. Evidence has shown that androgens are capable of binding to receptors in and around plasma membranes which activate cell signaling pathways that in turn elicit responses within seconds to minutes. Though much of this research has been demonstrated in estrogens [113, 114], there is growing evidence for similar effects from androgens [115].

A documented non-genomic effect of testosterone has been demonstrated with intracellular calcium regulatory mechanisms. Testosterone has been shown to affect calcium concentrations within neuroblastoma [116] and male osteoblast cells [117]. Furthermore, testosterone has been demonstrated to induce both vasoconstriction and vasodilation within various cardiovascular system structures, including the aorta and coronary arteries [118-123]. The relationship between testosterone and calcium regulation has also been proposed as a beneficial mechanism for improvements in sport performance; testosterone induced skeletal muscle sarcoplasmic reticulum calcium release may enhance the ability of explosive muscle contraction [124]. Additionally, androgen receptors have also been found to activate second messenger pathways independent of their transcriptional action within the cytoplasm [125] though it remains unclear as to whether these secondary pathways ultimately lead to transcriptional changes [126]. Regardless, the non-genomic action of testosterone provides insight into the possibility of more precipitous action on target cells.

Within the reproductive system, testosterone provides neuroendocrine regulation of GnRH through feedback. Specifically, high levels of testosterone inhibit LH secretion and vice-versa, a function that results from changes of pituitary sensitivity to GnRH [127]. However,
some findings have suggested that there is a neuronal component for androgen regulation of LH secretion [128-130] and though the specific sites are still unknown, this does open the possibility that testosterone may exert its effects on other neuronal regions such as the hypothalamus which, amongst other functions, regulates appetite and food intake [131].

![Diagram of Leydig cell testosterone synthesis](image)

**Figure 2.2**- Leydig cell testosterone synthesis. Synthesis requires conversion from cholesterol through a sequence of enzymatic steps. Cholesterol is incorporated into the cell by de novo synthesis from acetate or through extracellular supply via the LDL receptor. Cholesterol molecule’s side chains are shortened leading to production of pregnenolone within the mitochondria of the Leydig cell. Once formed, pregnenolone enters the endoplasmic reticulum of the Leydig cell and follows either the Δ4 or Δ5 pathway dependent on the location of the double bond in the pregnenolone steroid. The Δ5 pathway is more common in humans and is converted to the intermediate 17-hydroxy-pregnenolone which in turn is converted into dehydroepiandrosterone (DHEA). Both 17-hydroxy-progesterone from the Δ4 pathway and DHEA from the Δ5 pathway are used to produce the intermediate androstenedione, which is then reduced to testosterone by 17β-hydroxysteroid dehydrogenase.

**Testosterone and male puberty**

Testosterone is crucial in male pubertal development and production increases during normal male pubertal progression, exerting its effects on target organs critical for development.
Testosterone concentration correlates strongly with growth and growth velocity during pubertal onset. A 24-hour steroid profile of 41 pubertal males showed that growth velocity was significantly associated with morning testosterone levels ($r = 0.88, p < 0.001$) and when dividing the males into pubertal groups based on testicular volume, those with volumes indicating early puberty (3-6ml) showed the strongest association [8]. Given that peak growth hormone secretion is parallel to peak height velocity in males during mid-late puberty [132], it could be hypothesized that testosterone is critical for initial pubertal growth but is less important during later stages. The greater role of testosterone in early pubertal growth is supported by evidence showing weak correlations between testosterone and growth velocity during mid-late puberty [8]. Growth is primarily seen through the musculoskeletal system, and though testosterone does not seem to elicit a direct effect on bone growth, aromatization of testosterone into estradiol may be a critical step in bone development as estradiol plays an important role in bone formation [133] further discussed in 2.3.1.4.

Muscular growth during puberty is likely mediated through androgen receptors, which are located within both the muscle and the mesenchymal pluripotent muscle cells [134]. Testosterone binds to these receptors, translocating to the nucleus and binding to specific DNA sequences within the muscle [135]. From there, testosterone induces muscle fibre hypertrophy by acting at multiple steps in the pathways that regulate muscle growth. Testosterone stimulates pluripotent mesenchymal stem cells and commits them to muscle satellite cells, which increase in number [136, 137]. In addition, testosterone stimulates cell replication and increases myoblast activity while simultaneously down-regulating muscle protein degradation, which results in net gains of muscle tissue [138, 139]. Taken together, it appears that testosterone increases muscle volume as a result of myoblasts fusing to existing multinucleated muscle fibers called myotubes which then go through myogenic differentiation [134]. Along with this increase in muscular size, male puberty is also synonymous with secondary sexual characteristic development such as increased body hair and deepening of the voice.

During puberty, sexual maturation includes the production of axillary and pubic hair, which can be used as part of subjective measures of pubertal milestones [140]. Hair is produced by hair follicles which go through regular growth cycles that shed and grow new hair; a cycle hormonally regulated based on age/stage of development [141]. Androgens stimulate tiny fine colourless hairs (vellus follicles) forming longer and thicker pigmented hairs, particularly
in the axilla and pubis [140, 142]. These follicles must pass through a full hair cycle so that they may be shed and regenerate the lower follicle that will have more prominent changes associated with pubic hair growth development such as that on the upper pubic diamond, chest, and face [143]. During the latter stages of puberty, the male larynx goes through extensive changes as a result of increased testosterone, lowering the frequency of sound production; average adult male vocal frequency is 100 Hertz (Hz) compared to females who average 213 Hz [58, 144]. The growth of the larynx is primarily seen through enlargement of both the thyroid cartilage and vocal folds in addition to the lengthening of the vocal tract from androgen stimulation [145, 146].

Other manifestations of puberty include changes to the skin and acne. The appearance of substantial acne are noted during pubertal development in some males, and this is directly related to enhanced sebaceous gland activity, which have all the necessary enzymes for conversion of testosterone to 5α-dihydrotestosterone (5α-DHT) [147, 148]. Increased levels of testosterone are converted to 5α-DHT and bind to the nuclear androgen receptors of the sebaceous gland, where differentiation, proliferation and lipid synthesis occur [149].

2.3.1.4 Physiology of Estradiol and Role in Male Puberty

Estrogens are steroid hormones that are responsible for the development and regulation of female reproductive system in addition to female secondary sex characteristics development. There are three naturally occurring estrogens in human physiology; estrone, estradiol, and estriol. Of these, estradiol is the predominant hormone in terms of absolute levels and estrogenic activity. Estradiol in males is synthesized and secreted by testes and peripherally in the adrenal gland, where estradiol is converted from prehormones [150]. Additionally, desulphuration of conjugated estrogens in the liver provides added estradiol in males; estrone sulphate circulates in the blood and is converted by the liver into estrogen [151].

Synthesis of estradiol results from a side chain of cholesterol being cleaved from pregnenolone, which ultimately converts to androstenedione and testosterone. From there, androstenedione and testosterone are converted by specific isoenzymes and aromatase in both the gonads and peripheral tissues to convert to estradiol [152, 153].
Similarly to testosterone, 98% of plasma estradiol is bound to SHBG or albumin, and the remaining 2% is unbound [154]. Estradiol binds to an estrogen receptor, which changes shape, dimerizes, and interacts with DNA [155]. Though estrogen-receptor-α is the more common receptor and is expressed in the breast, uterus, cervix, and vagina as well as other organs, estrogen-receptor-β is more pertinent to males and can be primarily expressed in the prostate, testis, spleen, hypothalamus, and thymus [156]. Furthermore, sites such as the brain, bone, and vascular system have direct non-genomic estrogen action in the membrane [157]. There are four main mechanisms of estrogen signaling as described by Hall et al.[155]:

i) **Classical ligand-dependent**

Estradiol/estrogen receptor complexes (E2-ER) bind to DNA response elements (EREs) in target promoters, which results in an up or down-regulation of gene transcription followed by a response from the tissue.

ii) **Ligand-independent**

In the absence of estradiol, estrogen receptor function can be modulated by extracellular signals. Growth factors of cyclic adenosine monophosphate activate intracellular kinase pathways, which lead to phosphorylation and activation of the estrogen receptor at ERE-containing promoters without ligand/complex as described in the aforementioned classical signal.

iii) **ERE Independent**

E2-ER complexes alter the transcription of genes without ERE through association with other DNA-bound transcription factors.

iv) **Cell-Surface Signaling**

Unlike the other three mechanisms, this is a non-genomic signaling pathway. The three genomic pathways lead to mRNA that contain polysomes which allow for synthesis of proteins that give way to tissue responses. In contrast, cell-surface signaling involves estradiol activating a membrane-associated binding site that generates rapid tissue responses such as those seen in the brain, bone and vascular system [157].

*Estradiol and male puberty*
Morning values of 17β-estradiol were positively associated with both morning testosterone values and growth velocity in pubertal males [8]. Aromatase, an enzyme responsible for conversion of androgens into estrogens, was found to be deficient in some males and in addition to nearly undetectable estrogen levels, the individuals had low bone mass, unfused epiphyses, increased gonadotropins, and a poor lipid profile. These males also showed abnormal growth patterns during puberty and a larger than average body length due to unfused epiphyses [133, 158, 159]. This role of estradiol with epiphyseal fusion may in part also explain the rapid and earlier maturation of epiphyseal closure in girls [160].

One of the more critical functions of estradiol in males is bone growth and development. Previous work has shown that idiopathic osteoporosis in males is related to low peripheral estradiol levels [161] and bone acquisition in younger men is related to total and bioavailable estrogen levels, but not testosterone [162]. An interventional study in elderly men attempted to determine the effects of estradiol and testosterone on male bone formation. The males were rendered hypogonadal through administration of a GnRH agonist and an aromatase inhibitor which was followed by supplementation of testosterone, estrogen or a combination of both. Bone resorption was measured by urinary excretion of deoxypridinoline (Dpd) and N-telopeptide (NTx). Estradiol decreased Dpd and NTx excretion and, in comparison to testosterone, was more effective in increasing bone formation markers [163]. The authors concluded that although both sex hormones are important to bone formation, estrogen is the dominant sex hormone regulating bone resorption.

Estradiol may also play a role in reproduction and spermatogenesis as it aids in regulating the reabsorption of luminal fluid in the epididymis [164]. Research in animal models has demonstrated that there was excessive fluid accumulation in the seminiferous tubules of male mice with estrogen-receptor-β knockout, which consequently led to tubular atrophy and ultimately infertility [165].

2.3.1.5 Physiology of Sex Hormone Binding Globulin and Role in Male Puberty

Sex hormone binding globulin (SHBG) is a liver-derived glycoprotein that binds to sex hormones, specifically testosterone, estradiol and DHT [166]. SHBG is increased by thyroid hormone and estrogen, and it is decreased by androgens, growth hormone, insulin and glucocorticoids [154]. SHBG levels are unrelated to meals or the time of day [167]. Once
testosterone enters circulation into the blood, nearly half is tightly bound to SHBG and is considered to be unavailable for biological action on target cells [168], whereas albumin-bound and free testosterone can enter target cells through passive diffusion (2.3.1.3). Plasma SHBG is an important determinant in the amount of testosterone that can freely enter cells; small increases in plasma SHBG reduce the amount of circulating free testosterone [98].

There is a substantial rise in SHBG from birth to early childhood in both males and females, and during sexual maturation, a decline that is much more pronounced in males occurs due to high androgen levels [169]. Peripubertal obesity is associated with insulin-induced reductions in SHBG, which increases bioavailability of sex steroids including E2 [170, 171]. This reduction in SHBG leads to increase proportions of free testosterone resulting in increased clearance of testosterone from the body and a fall in production of testosterone and LH via the negative feedback mechanism [172].

2.3.2 Measurement of Puberty in Males

2.3.2.1 Clinical Staging

In a clinical setting, the Tanner Scale is used to assess pubertal maturity. The scale determines physical development using primary and secondary sex characteristics such as the testicular volume and pubic hair development in males and is based on a scale of 1 to 5 [140]. For testicular volume measurement, a Prader orchidometer is used to compare with palpated volume of the testicles, and this method is a valid and reliable measure of puberty [173, 174]. Tanner et al. [175] initially defined that the onset of male puberty is associated with a bone age (taken with the mean of several small bones of the hand and wrist) of approximately 13 years in boys. Though there is a fair amount of individual variation in addition to variation with different ethnicities, the approximate age of pubertal onset in boys is between 9 and 14 years of age [176]. An assessment using additional hormonal measures provides greater accuracy in determining pubertal stage. Hormonal measurements indicative of male pubertal progression (testosterone and LH), levels are measured fasting and in the morning since a diurnal and seasonal variation in the circulating testosterone levels exists [177].
2.3.2.2 Biochemical Measures

Testosterone

Due to the limitations of clinical staging, assessment of puberty can be further evaluated with the measurement of sex hormones pertinent to pubertal development. In adolescent males, the primary pubertal/sex hormones of interest are testosterone, SHBG, LH, and FSH. Of these, testosterone presents the greatest challenge in measurement due to methodological discrepancies and the lack of a recognized reference standard, particularly for low concentrations of testosterone seen in early adolescent males as well as high concentrations of interfering cross-reacting steroids [178]. These irregularities have ultimately led to a consensus statement [179] made by the Endocrine Society and the Center for Disease Control providing recommendations to improve consistency with testosterone measures in a clinical setting.

The most common methods of measuring testosterone are mass spectrometry (MS), and immunoassays (IA), including radioimmunoassay (RIA). MS works under the principle of a mass-to-charge ratio which is a dimensionless quantity formed by dividing the mass number of an ion by its charge number [180]. There are two forms of MS; gas chromatography (GCMS) and liquid chromatography (LCMS). To measure testosterone, a deuterium-labeled internal standard must be added to the sample since the stable isotope allows for precise measurement given the multiple steps involved in MS. Testosterone is then isolated and dried, and the sample is charged with electrons so that the molecules of testosterone are ionized. These ions are separated/deflected according to their mass-to-charge ratio by an electric or magnetic field; ions with the same mass-to-charge ratio will undergo the same amount of deflection, therefore, all the testosterone ions will aggregate together. A detector looking at charged particles of testosterone then identifies these ions [181].

An alternative to MS is measuring serum testosterone using IA, one form of which is RIA. This method has been in practice since the late sixties, with very few methodological changes since that time [182]. A known quantity of testosterone (antigen) is radioactively labeled and mixed with a known amount of antibody. The serum sample to be measured is then added and the antigen from this serum and competes with the radiolabeled antigen for binding sites. The more unlabeled antigen from the serum, the more it binds to the antibody and displaces
the radiolabeled antigen. The bound antigens are separated from the unbound ones, and a gamma counter is used to measure the radioactivity of the unbound antigens [183, 184].

Due to the complicated process of RIA, the most popular alternatives are non-radioactive labeled tracer IA’s such as chemiluminescent assay (CLIA) and enzyme-linked immunosorbent assay (ELISA). Much like the RIA, the known antigen is labeled and competes with the unknown serum antigen. In the ELISA, the tracer is represented by the antigen coupled by an enzyme that initiates a colorimetric reaction [185]. Specific to the testosterone antigen, colour density is inversely proportional to the amount of testosterone present and is read by a spectrophotometer. Similarly, the CLIA assay uses a competitive antigen labeled with a tag that emits light when bound. The sensitivity and reliability of this method is similar to RIA and much simpler to execute [186].

One of the fundamental challenges for researchers has been finding an accurate, sensitive and reliable measure of testosterone. Quite a few studies have compared IA vs. MS using relatively large sample sizes. Wang et. al. [187] compared both automated platform IA and RIA against LC/MS as the gold standard using samples from both healthy and hypogonadal adult males. Though there was a tendency to either overestimate or underestimate testosterone in the various IA and RIA analyses, they were generally acceptable for assessing testosterone values for adult males and identifying hypogonadism. Due to the lack of precision and accuracy of IA and RIA at lower serum concentrations of testosterone, they may not be as effective in measuring children and females. Similarly, other papers comparing MS to IA have concluded that IA tends to overestimate serum testosterone values in women and children [188, 189] where low values are expected. This overestimation may be due to the cross-reactivity with DHEA sulfate [190], though the exact cause remains to be determined. An analysis of first and second-generation testosterone IA’s compared against LC/MS indicated that all IA platforms showed good correlation with LC/MS when the testosterone concentration was >4.0nmol/l, but performed poorly when concentrations were below this value [191]. Tandem MS has consistently shown improved accuracy, requires low sample volume and is far more rapid and robust when compared to all other methods [192].

*LH & FSH*
Since an important marker of pubertal onset is pituitary gonadotropin activity, it is necessary to measure LH and FSH in conjunction with testosterone in males. Based on the biological profile of these glycoproteins, IA’s are the most common ways to measure LH [193-195]. Much like testosterone, various methods of IA’s have been used to measure gonadotropins since the 60’s. RIA’s for LH and FSH function similarly to that of testosterone where a radiolabeled LH or FSH preparation and serum LH or FSH compete for binding of the gonadotropin specific antibody [60]. Some of the limitations of RIA’s include the potential health hazards of dealing with radioactive tracers in addition to a short shelf life and high cost of equipment. Furthermore, there may indeed be issues with RIA capturing an accurate measure of gonadotropin molecules in low serum levels [196].

A non-competitive IA based on the sandwich technique is the most common form of measuring gonadotropins. This type of technique involves the use of two antibodies which bind to different sites on the antigen (gonadotropin). Two antibodies are added before and after the antigen (capture antibody and detection antibody respectively), and they bind to the antigen at different parts of the antigen (epitopes). The antigen is therefore ‘sandwiched’ between two antibodies, and as the antigen concentration increases, the amount of detection antibody increases, leading to a higher measured response [197]. In Immunofluorometric (IFIA) and CLIA’s, antibodies are labeled by linking to another chemical; a fluorescent label or a luminescent label allow for ease of identifying the quantity of the gonadotropins [194].

Similar to IFIA and CLIA’s, ELISA’s use a sandwich principle that was developed for the gonadotropins and was reproducible, highly specific and sensitive though showed limitations of measurement in prepubertal children [198]. Compared to RIA, ELISA techniques have several advantages, which include eliminating the problems associated with the use of radioisotopes, higher sensitivity, low inter-assay variation and simplicity/less working time per sample [194]. Since their initial development, the kits have improved with sensitivities of measuring serum gonadotropins [199], FSH and LH ten times higher than previous kits making it possible to obtain measurements at concentrations as low as 0.02 IU/L [200].

**Pubertal Assessment**

Some of the challenges of accurately assessing boys using self-staging may include variability of pubic hair growth in different ethnicities, over or underestimating genital size, and discordance between physical characteristics and gonadotropin production. Currently,
part of an accepted method of pubertal assessment in males is utilizing measurements of
testicular volume [59], performed by a clinical expert in this technique. However, this may
not be an accurate proxy in some males, including those that are obese who have shown
lower testosterone values for similar volumes to that of normal weight teenagers [31].
Similarly, in many pediatric studies, genital exams may reduce participant willingness to
consent and raises some concerns regarding feasibility. The main limitation with boys self-
staging is that it is problematic when compared to both trained pediatric endocrinologists
[31], and to actual hormonal derived pubertal assessment methods [201]; 39% of boys
incorrectly rated their pubic stage when compared to a trained endocrinologist [202].

Creating a Flow Chart for Pubertal Assessment

To evaluate pubertal stages based on a combination of hormonal measures and Tanner
staging in this study, various lab values have been estimated from multiple sources of
pubertal assessment literature [203-211]. A flow chart synthesizing this information for
guiding pubertal assessment using the hormonal measures of interest in conjunction with
testicular volume has been developed and presented in Appendix Figure A2.1. One of the
issues relevant when reviewing the literature relates to the sensitivity and cross-reactivity of
hormonal assays performed. For example, typical analyses that utilize direct assay
measurements (ELISA or RIA) have limited accuracy at both low and high testosterone
concentrations and lack standardization. Assays using a tandem MS or multiple-step IA
analyzers show improved sensitivity and specificity and are preferred for these measures in
boys, especially those with lower circulating levels of testosterone [212, 213]. Similarly,
gonadotropin assays have become much more sensitive with lower levels of detection able to
discriminate prepubertal from early pubertal boys. Thus, the results derived from these
studies were chosen based on use of sensitive assay measurements and MS methods were
weighted favourably due to the greater precision needed to detect low pre-pubertal hormonal
levels. In addition, the number of subjects measured, and method of Tanner staging for
comparison (i.e. self-staging versus clinician conducted testicular exam) was also taken into
consideration. Using the cumulative data to develop three tiers of analysis (LH, testosterone
& testicular volume), three proposed stages of puberty have been developed: i) Pre-Early
Puberty, ii) Early to Mid-Puberty and iii) Mid-Late/Post Puberty.

In a study by Chada et al. [204], 78 boys in various stages of puberty and pre-puberty were
assessed through genital Tanner staging while venous blood samples were taken for
measurement of testosterone, inhibin B, FSH and LH using ELISA. Based on this study, all boys with a LH value of ≤ 0.12 IU/L were pre-pubertal (Tanner stage 1). However, there was a considerable overlap of LH values between Tanner stage 1 and 2. LH values that fall between 0.12 and 0.44 IU/L are not specific enough and would indicate that additional measures such as testosterone and testicular volume need to be considered. There is an overlap between LH values of 1.05 and 2.39 IU/L during Tanner stage 3 (0.45-2.39 IU/L) and that of 4 and 5 (1.05-5.54 IU/L). Thus, incorporating the work of Resende et al. [210] where 59 pre-pubertal and pubertal males were divided by Tanner stage using a clinician to assess testicular volume and immunochemiluminometric and immunofluorometric assays, a Tanner stage 4 LH range was reported as 0.3-1.6 IU/L. Using the upper end of both Tanner stage 3 from Chada et al. and stage 4 from Resende et al, boys who have a LH value between 1.6 to 2.4 IU/L may be in either Tanner 3 or 4 stage.

Testosterone cannot be used as the primary surrogate of Tanner staging due to its high variability throughout puberty but can be used in combination with LH values (falling within the range of 0.12 - < 0.44 IU/L). Based on the work of Mouritsen et al. [214] and the established lower range of late pubertal males ages 14-16 and 16-19 from Konforte et al. [215], testosterone values that were shown to be ≤ 32-36 ng/dl would indicate the subject is pre-pubertal, thus ≤ 35 ng/dl is proposed as the cut off. If testosterone values are above this threshold (35-150 ng/dl), then a tertiary measure of testicular volume of < 3.0 ml could be included to define pre-early pubertal status and 3.0-11.0 ml to define early-mid puberty.

However, if the testosterone level is 410 ng/dl as determined by Kulle et al. [208] using LCMS, this will indicate that the boy is indeed in late puberty (Tanner 4 or 5). More recent data [214] utilizing a combination of pubic hair growth, testicular size and LCMS testosterone measures have indicated that the median testosterone concentration of testosterone increased twofold every 6 months from 12 months before onset of testicular growth (≥ 3ml) and that 6 months after the onset, testosterone ranged from 140-261 ng/dl. Based on the summation of this data, Mid-Late/post-puberty (Tanner stages 4 & 5) can be defined as an LH value of ≥ 2.4 IU/L, a testosterone value >150ng/dl and testicular volume > 12ml, as this seems to be both the lower end of adult testicular volume range and, in addition, indicative of maximum growth velocity in pubertal males [216].
2.4 Hormonal Appetite and Food Intake Regulation

The control of food intake (FI) and appetite comprises both short-term mechanisms and long-term signals of energy balance that involves central and peripheral systems working in concert with dietary components and FI [217]. An example of this includes the reduction of subsequent FI. Short-term control of FI occurs after a coordinated post-prandial response involving mechanoreceptors in the gut, nutrient concentration changes in the blood and release of post-prandial anorectic gut hormones [218]. This section will briefly discuss the physiology and mechanisms of some of the important central and peripheral hormones and systems involved in regulating appetite and FI.

2.4.1 Central Appetite and Food Intake

The arcuate nucleus (ARC) within the hypothalamus is critical in appetite regulation. The ARC is situated close to the median eminence where an incomplete blood-brain barrier may allow for peripheral signals to enter the central nervous system [219]. Situated within the ARC are a group of neurons responsible for control of FI whose axons project from the ARC to other hypothalamic nuclei. Neuropeptide Y (NPY) and agouti-related peptide (AgRP) enhance FI when activated (orexigenic). In contrast to these, pro-opiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART) act to coordinate a decrease in appetite and FI. POMC is the precursor to α-melanocyte stimulating hormone (α-MSH) which signals to decrease FI via the melanocortin receptors [131, 220].

The brainstem dorsal vagal complex (DVC) also plays an important role in appetite/FI regulation. The DVC plays a critical role in communication between the peripheral signals of FI (see 2.4.2 – 2.4.11) and the aforementioned hypothalamic nuclei [221]. This communication may be possible due to both an absence of a complete blood-brain barrier within the area postrema of the DVC in addition to neural projections from the brainstem to the hypothalamus [222]. Furthermore, vagal nerve afferents have been shown to carry information from the gut directly to the nucleus of the tractus solitarius within the DVC [223].

2.4.2 Ghrelin

Ghrelin is a 28 amino-acid-long gut peptide derived primarily from the stomach in addition to the proximal small intestine and pituitary gland. It activates NPY neurons in the ARC
stimulating appetite in addition to being a growth hormone secretagogue [224]. Ghrelin has been shown to significantly increase appetite and FI; it increases before a meal and decreases after a meal [225-227]. During fasting, ghrelin mRNA expression increases, and peptide levels of ghrelin return to baseline after feeding [228]. In rodents, central ghrelin administration induces FI [229] and peripheral ghrelin administration in healthy lean and obese individuals increases FI, induces the sensation of hunger and increases neural activation of specific regions of the brain associated with reward [227]. Furthermore, ghrelin decreases insulin secretion and increases gastric acid secretion [230] in addition to stimulating gastric motility and increases feeding frequency with no effect on meal size [225, 229, 231, 232].

2.4.3 Insulin

Insulin is a peptide hormone produced by the pancreatic beta cells, which regulates metabolism of carbohydrates and fats. Animal research has provided much insight into the effects of insulin on appetite. In rat models, central infusion of insulin resulted in decreased intake of food [233, 234], and conversely, infused antisense oligodeoxynucleotide (generated to prevent insulin receptor proteins from developing in the cerebral ventricle of the rat brain) resulted in hyperphagia and subsequent fat mass [235]. In adult humans, a meta-analysis performed with 92 normal weight and 44 overweight subjects showed that blood glucose might be an important satiety signal of short-term appetite regulation in normal-weight but not overweight subjects [236].

2.4.4 Leptin

Leptin, a product of the human leptin gene, is composed of 167-amino acids and is secreted and stored primarily in white adipose tissue, showing a positive correlation with white adipose tissue volume [237, 238]. Leptin acts on specific (ObRs) receptors in the brain located in nuclei of the arcuate nucleus (ARC), ventromedial (VMH), dorsomedial (DMH), and lateral (LatH) hypothalamus [239]. Several signal transduction pathways critical for regulation of energy homeostasis, FI, and glucose homeostasis are activated which includes Janus Kinase-Signal Transducer and Activator of Transcription-3 (JAK-STAT3) and Phosphatidylinositol 3-Kinase (PI3K) [240, 241].
In humans, the most significant roles of leptin are regulation of energy homeostasis and metabolism [242]. Leptin circulates throughout the body and serves as a measure of energy reserves in adipose tissue to guide central nervous system regulation of FI. Within the hypothalamus, leptin binds to ObRb receptors and inhibits a neural circuit that inhibits the appetite-suppressing POMC while activating the appetite-inducing NPY neuropeptides [131]. In addition to hypothalamic regulation of FI and appetite, leptin is also involved in the mesolimbic dopamine system, critical for motivation and reward of feeding [243].

### 2.4.5 Peptide YY

Peptide YY (PYY) is an anorexigenic 36 amino-acid peptide that is secreted by the L-cells of the distal gastrointestinal tract primarily from the ileum in addition to the colon and rectum [244]. The two endogenous forms, PYY_{1-36}, and PYY_{3-36} are released postprandially from L-cells after FI in humans and decrease FI [245, 246]. PYY_{3-36} is further produced by cleavage of the Tyr-Pro amino terminal residues of PYY_{1-36} by the dipeptidyl peptidase IV enzyme (DPP-IV). This allows for hypothalamic Y2 receptor selectivity [247]. PYY_{1-36} is predominantly circulated in the fasted state whereas PYY_{3-36} largely circulates postprandially [248].

Following FI, there is a marked increase of PYY_{3-36} within 15 minutes, reaching a postprandial peak at approximately 90 minutes. This peak may be elevated for up to 6 hours [245, 249]. The rapid post-meal increase of PYY_{3-36} indicates that initially, the release may be under neural control as the meal has not likely yet reached the ileum of the distal small intestine. More PYY_{3-36} is released thereafter when nutrients appear in the distal small intestine [250].

High circulating levels of PYY_{3-36} have been demonstrated in individuals with anorexia nervosa, in contrast to obese patients who have frequently shown low levels and a blunted postprandial response [251, 252]. Furthermore, in humans, peripheral administration of PYY_{3-36} has been shown to reduce FI by diminishing rewarding aspects of food in the brain [253, 254].
2.4.6 Glucagon-like-peptide-1

GLP-1 is a peptide produced and released postprandially by the intestinal L-cells (like PYY) [255] and acts on the hypothalamus [256] to decrease FI in both normal and overweight humans in addition to acting as an incretin and stimulating approximately 60% of the postprandial insulin secretion [257]. When stimulated, GLP-1 receptors, found in the central nervous system, have been shown to directly reduce both hunger and FI [258, 259], though the effect of GLP-1 on the expression of appetite mediating peptides AgRP, POMC, CART, and NPY is not completely known [260]. Secretion of GLP-1 is biphasic; an early peak occurs within minutes of FI as a result of neurohormonal reflex and direct nutrient exposure of proximal L-cells, a later peak from nutrient stimulation of distal L-cells [244]. In addition to central mediation of appetite and FI, GLP-1 plays an inhibitory role in gastric emptying through the ileal break which regulates the passage of nutrients [261-263].

2.5 Appetite and Puberty

2.5.1 Clinical Feeding Trials in Adolescents

Anecdotally it has been reported that boys ingest a high number of calories during puberty. In a study exploring short-term FI, Shomaker et al. [264] tested 103 males and 101 females between 8 and 17 across the weight spectrum by providing two identical lunch buffet meals following a standardized breakfast. Males ate more than females at all stages of puberty (including pre-pubertal) and FI increased across puberty. However, when the researchers adjusted for body composition (fat mass and fat-free mass), height, overweight status, and meal instruction, the main effect of sex remained significant. Since sex was shown to be an important contributor to larger FI in males, the male sex hormone testosterone may contribute to differences in FI compared to females.

2.5.2 Appetite Hormones and Puberty

A paucity of data exists demonstrating the relationships of appetite hormones with sex hormones and male puberty. Pubertal status affects appetite and appetite hormone secretion [265-267]. During puberty, plasma ghrelin decreases throughout developmental stages assessed by Tanner staging [268]. It remains to be seen if ghrelin is indeed a determining
factor in FI behaviour. Though fasting total and deacylated ghrelin were lower in pubertal boys who are overweight in comparison to boys who are normal-weight in similar pubertal stages, meal induced suppression of ghrelin was blunted in this population [269]. Furthermore, mean ghrelin concentrations of boys in later stages of puberty were lower in comparison to those in Tanner stage 1, and fasting blood concentrations of acylated and non-acylated ghrelin are higher in pre-pubertal boys when compared to those in puberty [270, 271].

Anorexigenic peptides have also shown relationships with male pubertal development. In adolescent boys, those who were overweight tended to have lower fasting and postprandial GLP-1 in comparison to normal-weight boys [272]. In addition, PYY was inversely related to growth hormone secretion during varying stages of puberty which could be beneficial during growth (lower postprandial satiety resulting in more nutrients ingested for growth) and it was shown to be lowest in mid to late pubertal boys [273]. The increased growth hormone secretion is synonymous with puberty and is in direct relation to pubertal insulin resistance.

Insulin resistance in boys increases significantly between early puberty to mid puberty (Tanner 2 to Tanner 4) and decreases to near prepubertal levels in Tanner stage 5 independent of fatness and body mass index (BMI) [274]. It has been proposed that this increase in insulin resistance may result from increased production of growth hormone and subsequent peripheral increases of IGF-1, as resistance has shown a positive correlation with serum IGF-1 levels and mean serum GH levels [275, 276].

The aforementioned hormones are all implicated in short-term appetite signaling with puberty, but leptin, a chronic mediator of appetite has indeed shown relationships with pubertal development. Leptin is an important signalling molecule involved in the timing of onset of pubertal development [277]. In boys, rising serum leptin levels precede the onset of puberty and decrease immediately after initiation of puberty even after adjusting for body composition [278, 279]. The sexual dimorphism of leptin throughout pubertal progression (decreasing in boys and increasing in girls) implicates a role of gonadal steroids as mediators in circulating leptin [280].
2.5.3 Appetite Hormones and Overweight/Obesity

In contrast to pubertal development, a great deal of work aiming to determine the relationship of appetite hormones in overweight/obese individuals has been performed. In obese boys, short-term FI is affected by body fatness [281]. Obese individuals also demonstrate ghrelin levels which are lower than normal weight subjects during periods of weight stability, though ghrelin does increase during diet-induced weight loss [266, 282]. Furthermore, there does not seem to be a resistance to ghrelin as previously thought [283]. Though it seems that ghrelin signaling follows what would be expected for adiposity/energy availability, less is clear about anorexigens.

Circulating postprandial levels of PYY are lower in obese individuals [246], and postprandial stimulation of PYY may be blunted in obese adolescents [269]. However, results from our laboratory [266] comparing normal weight and obese adolescent boys contradict the findings of lower circulating PYY in obese adolescents and showed that PYY AUC is significantly higher in obese boys. Boys with obesity within our prior study protocol had a lower whole-body insulin sensitivity index (WBISI) in comparison to normal weight boys (5.5 ± 1.7 and 11.4 ± 1.8) respectively as well as greater fasting insulin resistance (HOMA-IR) values (3.2 ± 1.0 vs. 0.9 ± 0.2 respectively) implicating insulin resistance within the cohort of obese boys. Previous literature has indicated that there is in fact a high level of serum PYY in obesity associated insulin resistance and type 2 diabetes [284]. Since insulin resistance is related to incretins, it would be expected that the anorexigenic incretin GLP-1 would be affected by obesity. Obesity has been shown to be associated with a decreased postprandial GLP-1 response to feeding [285, 286]. Administration of GLP-1 analogs to obese patients decreased bodyweight and improved blood glucose regulation and insulin sensitivity [287, 288].

It is well established that obesity is associated with insulin resistance, though there is still debate with regards to the primary underlying mechanisms [289]. In adult humans, a meta-analysis performed with 92 normal weight and 44 overweight subjects showed that blood glucose may be an important satiety signal for short-term appetite regulation in normal-weight but not overweight subjects [236]. This discrepancy in appetite signaling may be a function of increased insulin levels leading to increased satiety levels in normal-weight subjects which could also be impaired in overweight subjects due to central insulin resistance. In children with overweight, insulin resistance and hyperinsulinemia has been associated with increased energy intake during an ad libitum buffet after an overnight fast a 10% increase in
HOMA-IR was associated with 29 kcal greater energy intake [290]. During pubertal development, obese children had larger increases of insulin resistance (measured by HOMA-IR) than normal-weight children and, additionally unlike the normal-weight children, did not return to normal values after puberty [291].

Much like insulin, individuals with higher levels of adiposity have greater circulating leptin levels when compared to normal-weight subjects [292]. However, the satiety promoting properties of leptin seem to be attenuated as a result of leptin resistance in overweight and obese patients, though it remains to be seen whether the resistance is a function of the excess body fat or, whether leptin resistance predisposes an individual to greater FI resulting in increased adiposity [293]. Given that leptin and insulin act upon the same hypothalamic areas to suppress FI [294, 295], central resistance to both hormones in the hypothalamus may be related and an important factor in obesity and appetite regulation.

2.5.4 Testosterone and Overweight/Obesity

The inverse relationship between body weight and testosterone levels in males has been well documented [296-299]. Testosterone may also be predictive of weight regain after weight loss in males [300], and severe childhood obesity is associated with impaired Leydig cell function in young men [301]. Though some controversy exists, pubertal boys with obesity have lower SHBG and lower total testosterone in comparison to normal-weight boys [302], with increased aromatization due to high adiposity advancing skeletal age in overweight/obese boys [303]. The relationship between body weight and testosterone seems to be specific to adipose tissue and body fat as demonstrated in comparisons of younger and older men [304]. Further studies looking at both healthy and hypogonadal men showed that the latter had increased fat mass in addition to increased abdominal/central obesity [305, 306]. Similar findings in pubertal males indicated that adolescents with obesity had lower free testosterone levels and signs of Leydig cell impairment in spite of testicular volume being equivalent between boys with obesity and their normal weight peers [31].

Testosterone has been demonstrated to have direct physiological effects on adipose tissue deposition. Acetyl-CoA synthetase is a fatty acid (FA) activator which signals enhanced FA uptake in adipose tissue [307]. Testosterone may decrease expression of acetyl-CoA synthetase. A study of hypogonadal men showed that in comparison to eugonadal men, hypogonadal males had a propensity to store FA’s and FFA’s in lower subcutaneous fat
which was associated with increased expression of acetyl-CoA synthetase [308].

Lipoprotein lipase (LPL) is an extracellular enzyme which hydrolyzes circulating blood lipoproteins into FA’s which are taken up into the adipocyte and esterified into triglycerides that are then stored in the adipocyte [309]. In sedentary obese men, abdominal adipose tissue LPL activity was inversely correlated with bioavailable plasma testosterone [310].

A hypogonadal obesity cycle has been proposed by Cohen in male patients with obesity [311]. High aromatase activity in adipose cells converts testosterone to oestradiol and decreases tissue testosterone. This decrease in testosterone results in greater LPL activity which increases triglyceride uptake into the adipocyte. As well as adipocyte volume increasing, low testosterone stimulates pluripotent stem cells to be differentiated into adipocytes thus increasing both the size and number of these cells. The increased levels of oestradiol, TNFα and leptin from increased adipocyte cell mass prevent the hypothalamic-pituitary-testicular axis response to decreased testosterone levels [312]. This is a result of kisspeptin being both inhibited by the inflammatory cytokines as well as oestradiol and being resistant to leptin, thus preventing downstream signaling of GnRH and LH [313, 314].

Finally, in addition to partitioning greater fat oxidation within the mitochondria [315], testosterone enhances noradrenaline stimulated lipolysis which may be a result of testosterone increasing the number of β-adrenoceptors within fat cells [316, 317]. Collectively, testosterone helps mitigate adiposity in males and decreased levels of testosterone result in increased adiposity. This relationship is cyclical as an increase of adiposity decreases testosterone through both aromatization and greater clearance of SHBG [311, 318].

2.5.5 Testosterone and Food Intake/Appetite

Testosterone may influence hormones involved in appetite and vice-versa (Figure 2.3). During pubertal progression, increased testosterone and subsequent aromatization to estrogen has been shown to exert increases of pulsatile GH release, which in turn increases insulin-like growth factor 1 (IGF-1) [319]. Insulin-like growth factor 1 (IGF-1) is known to suppress PYY and there is an observed decrease of PYY during pubertal progression [273]. Thus, increased testosterone during pubertal progression is presumed to depress PYY indirectly by its stimulatory action on the somatotropic axis. Since PYY decreases during pubertal
progression in boys [273], it can be posited that this decrease of the anorexigen could in part play a role in the observed increases of FI in boys.

Another important link with appetite and sex hormones is one between testosterone and ghrelin. In boys, there is a marked decrease in ghrelin with increased pubertal stage [268]. Ghrelin has been detected in Leydig cells of the testes and is inversely correlated to testosterone in adult males [320]. In boys and adolescents, short peripubertal boys given therapeutic injections of testosterone had significant decreases in ghrelin [268]. Since ghrelin is considered a short-term regulator of feeding initiation, it is unknown whether this hormone plays a key role in overall FI of adolescent boys. Indeed, the inverse relationship between testosterone and ghrelin, a known GHRH stimulant, observed during pubertal progression, may simply result from a negative feedback loop due to increased GH/IGF-1.

Insulin promotes satiety by inhibiting arcuate nucleus expression in the neuropeptide-Y region of the hypothalamus, thereby decreasing FI [321, 322]. Insulin resistance increases during puberty by approximately 30% as a result of increases in GH/IGF-1 [275, 276]. Estrogen increases, in part due to aromatization of testosterone, also promote GH/pituitary secretion and insulin resistance [319]. Testosterone could therefore indirectly increase FI through pubertal insulin resistance mediated by GH/IGF-1. This is similar to what has been proposed with PYY.

Leptin is an essential hormone in energy homeostasis through its effects on inducing satiety [131]. Leptin is a critical hormone for normal pubertal development; it increases prior to pubertal onset and stimulates the release of GnRH [323]. Unlike girls, leptin in boys begins to rapidly decrease after the onset of puberty, irrespective of body composition [278]. This sex difference could relate to testosterone lowering plasma leptin, as shown in a study of hypogonadal adult men [324]. In a multiple regression analysis of factors related to puberty, leptin was inversely related to testosterone levels but not fat mass, fat-free mass or estrogen, LH and FSH levels [278, 279]. Thus, lower leptin may be another mechanism leading to an increased FI during puberty in boys. Leptin is important in both energy homeostasis and pubertal onset. Taken together, lower leptin could lead to an increased FI during puberty. Furthermore, leptin and insulin signaling pathways share an overlap in the ventromedial hypothalamic neuron and insulin resistance could interfere with leptin signal transduction [325], leading to an increase of FI.
Figure 2.3 - Proposed relationship between testosterone and food intake during puberty. Testosterone may indirectly increase food intake through aromatization resulting in a decrease of postprandial PYY\textsubscript{1-36} and PYY\textsubscript{3-36} (PYY) in addition to increased insulin and leptin resistance with a concomitant decrease of circulating leptin resulting in increased food intake during puberty.

Much like the research available in puberty and GLP-1, limited work has been performed on determining relationships between GLP-1 and testosterone. Previous research in animals has implicated GLP-1 in LH release by showing that cerebroventricular injections of GLP-1 increased plasma LH [326]. In addition, GLP-1 may play a role in decreasing testosterone pulsatility in humans through direct GLP-1 infusion. However, unlike in animals, this seems to be independent of LH [327]. Animal studies suggest that the down-regulatory effect of GLP-1 on testosterone may be explained by a neural pathway between the hypothalamus and testes which regulates secretion of testosterone independent of the pituitary [328]. The relationship between GLP-1, appetite, and testosterone during puberty in humans remains unclear as testosterone pulsatility increases during pubertal growth.

Testosterone’s relationship with appetite may not be limited to indirect effects via appetite hormones. As puberty advances, so too does FFM and FI [329] but even after adjusting for body composition, height, and weight status, short-term FI in 8-17-year-old males remained greater than in females [264]. Testosterone may have a direct effect on appetite and FI as
androgen receptors are located in the hypothalamic-pituitary-gonadal axis [330]. In animal studies, orchiectomy decreased FI [331, 332] and this effect was reversed through testosterone administration [331, 332] which has been previously shown to stimulate FI in adult rats with intact testicles [333].

Though testosterone does have a relationship with appetite and appetite-related hormones, there is also evidence to show that FI can affect testosterone levels. Chronic high protein intake in a group of males have been shown to reduce fasting testosterone concentrations [334, 335]. Furthermore, 6 weeks of meals containing animal fats attenuated the reduction of testosterone in comparison to meals that were either high in plant-based fats or low fat in adult males [336]. Less is known about acute FI and testosterone, though previous research has shown a decrease in serum testosterone in adult males immediately after glucose ingestion [10].

2.6 Testosterone and Exercise

The aforementioned literature focuses on the relationship between testosterone and energy intake, one half of basic human energy balance. Thus, it would be imperative to also discuss the relationship between testosterone and energy output in adolescent males.

2.6.1 Studies of testosterone change from exercise in males

In adult males, an increase of plasma testosterone levels has been shown as a result of both anaerobic and aerobic modes of exercise such as weight lifting [337] or cycling [338] and running [339] respectively. In contrast, studies of the effect of exercise on testosterone levels in adolescent males are far less abundant. Longitudinal studies in boys who are in Tanner stages 1 and 2 showed no significant change in testosterone during cardiorespiratory exercise (20 min cycle ergometer, 60% of VO2peak) and later when they were more mature, still showed no differences. However, testosterone in these boys was measured with radioimmunoassay which may not be sensitive enough to detect low levels in this cohort. Furthermore, no control/non-exercise sessions were performed to determine whether there were differences between exercise and regular diurnal rhythms [340].
Research examining the effect of exercise in boys has focused primarily on longitudinal interventions and aerobic style exercise programs and it has been observed that training in young individuals may modify sex hormone responses at rest and during exercise. Cacciari et al. [341] reported that in 175 trained male soccer and untrained males aged 10 to 16 years, the plasma level of DHEA-S was significantly higher in trained children. This enhanced plasma DHEA-S concentration was related to higher levels of testosterone. Mero et al. [342] described serum testosterone levels under conditions of rest before, during and after a year of long distance running, sprint running, weightlifting and tennis training in boys aged 10 to 12 years. Compared with untrained boys, the initial plasma testosterone concentration was almost 3 times higher in these individuals. Furthermore, the mean testosterone level was approximately doubled after a year of training. These results suggest that endurance training may alter gonadal hormone production in young athletes.

Fewer studies have examined the acute effects of exercise on testosterone in adolescent males. Klentrou et al.[343] studied the effects of anaerobic style exercise training on male athletes ages 12-14. The subjects were asked to perform 30 minutes of resistance or plyometric exercise compared with a 30-minute control session. The results of this study showed that testosterone increased from pre-exercise to post exercise in both the resistance and plyometric modalities. Other studies looking at acute testosterone levels in males found similar results with anaerobic protocols such as weightlifting [344] and sprinting [345]. In one of the few aerobic studies observing testosterone change, Hackney et al. presented the effect of aerobic style exercise training in males who were in Tanner stages 4 and 5. The participants performed 20 minutes of incremental exercise to exhaustion using a cycle ergometer. Results indicated that there were no significant differences between pre- and post-exercise testosterone levels. The lack of control session was a major limitation of the Hackney study. Additionally, the males were only fasted for three hours prior to testing which could impact the baseline testosterone levels. Finally, because testosterone was measured using immuno-chemoluminescence, sensitivity to detect small changes may be an issue [346]. Given that there is little research in comparison to anaerobic protocols, further work on the effects of aerobic exercise on acute testosterone levels in adolescent males using appropriate controls should be performed.
2.6.2 Physiology of testosterone in exercise

Though there is indeed a marked increase of testosterone from exercise in adult males and possibly adolescent males, the physiological mechanisms behind these phenomena are less clear. Several studies using different forms of exercise have repeatedly shown that the level of LH in plasma does not change after exercise [11-13] indicating that the increase of testosterone from exercise is not centrally mediated and exercise itself may stimulate peripheral changes of testosterone production.

With both anaerobic and aerobic physical exertion, there is an increased production of lactate as a result of glycolysis and glycogenolysis within skeletal muscle tissue [347]. Lin et al. discovered that in male rats, infusion of 5-20mM of lactate resulted in a dose-dependent increase of testosterone production by 25-OH-choesterol in the Leydig cells. It is thus hypothesized that lactate directly affects the Leydig cell cAMP production of testosterone and that P450scc is a target of lactate [14]. Further studies have confirmed that exercise-induced lactate production resulted in dose-dependent increases in testosterone and testicular cAMP in rats [348]. Thus, it seems that there is a concomitant increase of testosterone and lactate production during exercise, but this has yet to be validated in humans.

2.7 Summary and Research Rationale

It is clear that puberty is a time of adjustment of FI regulatory mechanisms and presents the risks of setting a course for increased FI and obesity. However, unlike obesity, the changes in pubertal FI may be a consequence of the increases of sex hormones rather than the homeostatic imbalances seen with obesity. For boys, testosterone plays a major role in growth and development. What is currently unknown is (a) whether the relataionship between testosterone and FI differs in boys with obesity vs. pubertal boys of normal weight, (b) whether acute changes of testosterone correlate with FI in pubertal boys across the weight spectrum, (c) how food and macronutrients affect acute testosterone changes, and (d) how testosterone affects the postprandial response of appetite hormones. Furthermore, it remains unclear what the response of testosterone is after aerobic exercise in this population and whether this response is related to appetite and FI.
Therefore, the objective of this thesis is to determine how testosterone is affected by food and exercise and describe the relationship between testosterone, FI and appetite in adolescent males. It is expected the results of this thesis will add to current knowledge of testosterone by providing new insights into the role of this hormone in appetite and FI.
Chapter 3

HYPOTHESIS AND OBJECTIVES
3 HYPOTHESES AND OBJECTIVES

3.1 Overall Hypothesis and Objective

Objective

- To determine the interactions between testosterone responses to food intake and exercise with appetite and food intake in adolescent males.

Hypothesis

- Testosterone responds to food intake and high intensity exercise and has an interactive relationship with the short-term regulation of appetite and food intake in adolescent males.

3.2 Specific Hypotheses and Objectives

Chapter 4: ACUTE DECREASE IN SERUM TESTOSTERONE AFTER A MIXED GLUCOSE AND PROTEIN BEVERAGE IN OBESE PERIPUBERTAL BOYS (published in Clinical Endocrinology 2015, 83: 332-338).

Objective:

- Explore the effect of a mixed glucose/protein beverage on acute endogenous testosterone levels in overweight adolescent males.

Hypothesis:

- A mixed glucose/protein beverage will decrease endogenous testosterone levels an hour after ingestion in adolescent overweight males.

Chapter 5: ACUTE DECREASE IN PLASMA TESTOSTERONE AND APPETITE AFTER EITHER GLUCOSE OR PROTEIN BEVERAGES IN ADOLESCENT MALES (published in Clinical Endocrinology 2019, 83: May 4th)

Objective:

- Compare the effect of glucose or protein provided at 1g/kg body weight in a beverage form on acute responses in plasma testosterone and appetite hormones, appetite, and food intake in adolescent males 12 to 18 years of age.
Hypothesis:

- Postprandial testosterone levels play a role in the regulation of short-term appetite and food intake in teenage boys.

Chapter 6: ACUTE DECREASE IN APPETITE IS UNRELATED TO INCREASE IN PLASMA TESTOSTERONE DURING HIGH INTENSITY CYCLING IN ADOLESCENT MALES

Objective:

- Determine whether high intensity aerobic exercise will increase testosterone and decrease appetite in adolescent males and define whether the responses are related.

Hypothesis:

- Three 10-minute bouts of high intensity recombinant cycle exercise at 75% VO2peak will increase testosterone levels and decrease appetite in adolescent males but the responses will be related.
Chapter 4

ACUTE DECREASE IN SERUM TESTOSTERONE AFTER A MIXED GLUCOSE AND PROTEIN BEVERAGE IN OBESE PERIPUBERTAL BOYS

The following chapter is a reproduction of a manuscript that has been published in Clinical Endocrinology 2015 Sept; 83 (3): 332-338
ACUTE DECREASE IN SERUM TESTOSTERONE AFTER A MIXED GLUCOSE AND PROTEIN BEVERAGE IN OBESE PERIPUBERTAL BOYS

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4.1 Abstract

Background and Objectives Delayed puberty and lower levels of testosterone (T) have been observed in adult obese males and some adolescent males. In adult men, enteral glucose ingestion results in acute lowering of serum testosterone levels, however this has not been studied in adolescents. We aimed to examine the acute effect of a glucose/protein beverage on serum T concentration changes in obese peripubertal males. A second objective was to determine whether change in T concentration was related to appetite hormone levels.

Patients and Methods 23 overweight and obese males age 8-17 in pre-early (Tanner stage 1-2) and mid-late (Tanner stage 3-5) puberty were included in this cross-sectional study at the Clinical investigative unit at the Hospital for Sick Children. Participants consumed a beverage containing glucose and protein and blood samples measuring pubertal hormones, ghrelin and glucagon-like-peptide-1 (GLP-1) were taken over 60 minutes.

Results Across pubertal stages, there was a significant decrease in T levels in adolescent boys (-18.6 ± 3.1%, p < 0.01) with no proportional differences between pre-early and mid-late puberty (p =0.09). Decrease in T was associated with a decrease in LH (r = 0.52, p = 0.02) and fasting T was inversely correlated to fasting ghrelin (r = -0.51, p = 0.03) with no correlation to GLP-1.

Conclusions Intake of a mixed glucose/protein beverage acutely decreases T levels in overweight and obese peripubertal boys. A potential mechanism for this decrease may be secondary to an acute decrease in LH but this requires further evaluation.
4.2 Introduction

Obesity is associated with a reduction in serum total testosterone levels in adult men [349]. Testosterone, a steroid hormone which plays a key role in male pubertal development and secondary sexual characteristics, is 40-50% lower in obese boys when compared with normal weight boys [9] and may be associated with pubertal delay in overweight and obese boys [27].

Several mechanisms have been proposed to account for the inverse association between obesity and testosterone levels. Androgens are converted to estrogens via an aromatase enzyme present in adipocytes, lowering the testosterone: estrogen ratio [311]. Obesity is also associated with chronic hyperinsulinemia and insulin resistance [350] which in turn, may lower sex hormone binding globulin (SHBG) resulting in lower levels of circulating testosterone as well as an increased clearance of testosterone [351]. Furthermore, excess adiposity results in a greater production of the energy homeostasis-regulating hormone leptin leading to central leptin resistance at the level of the hypothalamus, which may decrease the production of gonadotropins and testicular testosterone production [293, 314, 352]. Finally, a small number of studies in adult males suggest that testosterone may also be affected by acute glucose intake. In these studies, direct parenteral insulin and glucose infusion led to increased testosterone levels [353, 354]; however, enteral ingestion of a glucose beverage acutely lowered testosterone levels [10]. Reasons for this discordance are unclear yet may relate to the effect of gut hormones released with enteral intake, such as glucagon-like peptide-1 (GLP-1) and ghrelin which have been shown to decrease testosterone levels and pulsatility in animals and healthy adult males [327].

GLP-1 is secreted by the L cells of the intestines in response to feeding and is responsible for increasing insulin release and inducing satiety centrally [355]. In one study performed in adult males, GLP-1 infusion during an intravenous glucose challenge resulted in decreased testosterone pulsatility implicating a down-regulatory effect of GLP-1 on testosterone [327]. Ghrelin is a 28 amino-acid-long gut peptide derived from the stomach in addition to the proximal small intestine and pituitary gland. Ghrelin activates neuropeptide-Y neurons in the arcuate nucleus to stimulate appetite [224] and has been shown to rise acutely prior to feeding initiation and decrease following food intake [227]. In boys, fasting ghrelin levels decrease with increased pubertal stage [356]. Ghrelin is also inversely correlated to testosterone in
adult males and has been detected in Leydig cells of the testes, which are responsible for testosterone production, although its role in the testes remain unclear [320].

The interplay between testosterone, glucose, and other hormones related to acute food intake has not been assessed in peripubertal obese boys. Accordingly, the primary objective of this study was to determine the acute effect of a glucose/protein beverage on testosterone levels in obese adolescent males across different pubertal stages. A secondary objective was to explore the relationship of testosterone following the beverage to degree of adiposity, insulin sensitivity, GLP-1 and ghrelin levels.

4.3 Materials and Methods

4.3.1 Subjects

Overweight and obese but otherwise healthy adolescent boys age 8 to 17 were recruited as part of a larger study of girls and boys across the weight spectrum exploring childhood and adolescent obesity (HISTORY: High Impact Strategies Towards Overweight Reduction in Youth) and the appetite hormone response related to clinical pubertal status [357]. Recruitment occurred via flyers posted in the hospital, and a print ad in the local newspaper. All eligible overweight and obese boys were included in the analysis. Obesity was defined using the Center for Disease Control BMI charts where overweight is categorized as 85th-95th percentile and obesity is categorized as ≥95th percentile [21]. Subjects were excluded if they had a history of prematurity, chronic illness or were taking any medications known to affect glucose homeostasis, appetite or pubertal development. The results presented in this paper are based on a secondary analysis of a previous study to examine appetite hormones in response to a mixed glucose-whey drink [357]. The study was approved by the University of Toronto Research and Ethics Board for Humans and The Hospital for Sick Children Research Ethics Board.

4.3.2 Protocol

After a 10-12 h fast, participants arrived at 0800 and were administered a motivation-to-eat VAS scale, which measure dimensions of subjective appetite and have been previously validated for use in boys after glucose preloads [281]. The scale was composed of four questions: (1) How strong is your desire to eat? (“very weak” to “very strong”), (2) How
hungry do you feel? (“not hungry at all” to “as hungry as I’ve ever felt”), (3) How full do you feel? (“not full at all” to “very full”), and (4) How much food do you think you can eat? (prospective food consumption, PFC) (“nothing at all” to “a large amount”). Participants were instructed to read each question and place an “x” along the 100 mm line depending on their current feelings. An intravenous catheter was placed for blood sampling.

Participants were then administered a mixed beverage containing 30 g glucose monohydrate (Grain Process Enterprises, Toronto, ON Canada) and 30 g of whey protein isolate (plain whey-protein isolate; Interactive Nutrition International Inc., Ottawa, Ontario, Canada) plus aspartame-sweetened, orange-flavored crystals (1.1 g, Sugar Free Kool-Aid, Kraft Canada Inc., Don Mills, ON Canada) to standardize flavor. The combination of a standardized glucose load and protein has been shown to generate a greater insulin increase and ghrelin decrease in comparison to a glucose load only potentially through protein-stimulated GLP-1 secretion [358, 359]. Thus, this combination was chosen to promote a greater change in hormone flux. The beverage was consumed within 5 min, followed by 50 mL of water to minimize aftertaste.

Blood samples were taken of testosterone, LH, FSH, GLP-1 and ghrelin at fasting and at 15, 30 and 60 minutes after completely ingesting the mixed beverage. 15 and 30 minutes were chosen in order to capture the rapid postprandial rise (GLP-1) and fall (ghrelin) of these peptides in the circulation [355, 360]. The 60 minute time point was chosen as a previous study in adults has indicated post-glucose load nadir testosterone values at this time [354].

In a separate visit conducted within 4 weeks of the visit described above, body composition was analyzed via BOD POD (Life Measurement Incorporated, Concord, CA) air-displacement plethysmography (ADP) [361]. A standard, calibrated scale and wall-mounted stadiometer were used to measure weight and height, and body mass index (BMI) calculated as weight (kg)/height (m)^2. Three trials of these measurements were completed and the mean was taken for analyses. Puberty was assessed through a validated Tanner staging questionnaire and via examination by a paediatric endocrinologist [362]. A multiple-sampled (0, 30, 60, 90 and 120 minutes) oral glucose tolerance test (7 1.75g/kg to a max of 75g glucose) was performed to assess insulin sensitivity [363]. Insulin sensitivity was calculated by the Matsuda Model whole body insulin sensitivity index (WBISI) where WBISI = \((10,000/\text{square root of } [\text{fasting glucose } \times \text{ fasting insulin}]) \times [\text{mean glucose } \times \text{ mean insulin during OGTT}]\). Assessment of normal glucose tolerance, impaired fasting or 2-hour glucose
and type 2 diabetes was completed by measurement of the 0 and 120-minute glucose levels using the Canadian Diabetes Association Clinical Practice Guidelines [364].

4.3.3 Biochemical Assays

Glucose was analyzed using Glu Microslides, Vitros 950 chemical system (Ortho Clinical Diagnostics). Insulin was measured using a chemiluminescence immunoassay (Siemens Immulite 2500 platform; range 15–2165 pmol/l, intra- and inter-assay coefficient of variation [CV] <7.6%). Human active GLP-1 (intra-CV: <8%; inter-CV: <5%; #EGLP-35K), total and acylated ghrelin (intra-CV: <2%; inter-CV: <8%; #EZGRT-89K) were measured with ELISA kits (Millipore, Billerica, MA, USA). Samples were stored at -80ºC until analysis, and all samples were run in duplicate.

FSH was assayed to ensure that none of the males had evidence of primary testicular insufficiency (indicated by an elevated FSH) using a chemiluminescent assay platform (Abbott ARCHITECT ci8200) used for clinical and research purposes at the Hospital for Sick Children. The same platform was also used to measure testosterone and LH (testosterone sensitivity 0.3 nmol/L and cross reactivity of 0.01-2.11% for various metabolites; LH sensitivity of 0.07 IU/L and cross reactivity of 0.84% for TSH with no cross-reaction to FSH or hCG).

4.3.4 Statistical Analyses

Sample size was assessed using PS Power and Sample Size Calculations (Version 3.0.43, 2009, Vanderbilt University). The mean of three adult studies showed decreases of testosterone of 24% following an OGTT [10, 365, 366]. Based on this estimated relative decrease, and using mean and standard deviation values from studies measuring testosterone in boys from T1 to T5 puberty, 13 boys were required to achieve power of 0.8 and an α = 0.05 [208].

A two-way repeated measured ANOVA was used to analyze the effects of puberty and time and their interactions on testosterone before and after the glucose and protein beverage. A Tukey’s test post-hoc analysis was then performed. Pearson correlation was performed to evaluate testosterone (baseline) and change in testosterone to measures of adiposity, insulin
sensitivity (WBISI), and change in LH, ghrelin and GLP-1. All analyses were performed with Statistical Product and Service Solutions software, version 17.0 (SPSS Inc., Chicago, IL). Effects were considered significant at p < 0.05 and data are presented as mean ±(SEM).

4.4 Results

Subject characteristics are presented in Table 4.1. A total of 23 boys with a mean age of 13.3 ± 0.6 years and mean BMI of 33.1 ± 2.2 kg/m² participated. 10 boys were classified as being in the pre-early stages of puberty (Tanner stage 1-2) while the remainder (n = 13) were in the mid-late stages of puberty (Tanner 3-5) mean WBISI values of 3.3 ± 1.1 and 3.4 ± 0.8 respectively. All were normoglycemic with the exception of two boys in mid-late puberty who exhibited impaired glucose tolerance on the 2-hour OGTT.

Fasting testosterone levels significantly differed between pre-early and mid-late puberty (2.3 ± 0.7 vs. 9.9 ± 1.6 nmol/L respectively, p =0.007). Fasting testosterone levels were inversely correlated to body fat percentage (r= -.49, p = 0.02) but not to BMI Z-score (r= -0.21, p = 0.34) or WBISI (r= .21, p = 0.34). There was a significant inverse relationship with fasting testosterone levels and fasting active ghrelin prior to ingestion of the beverage (Fig. 4.1). After controlling for percentage body fat, there remained a trend in this relationship; however, it was no longer statistically significant (r = -.46, p = 0.06).

For the group as a whole, testosterone levels decreased significantly by 1.9 ± 0.4 nmol/L 30 minutes after ingestion of the glucose and protein beverage (p < 0.01). At 60 minutes after ingestion of the beverage, testosterone levels remained significantly lower than baseline at 1.6 ± 0.3 nmol/L (p < 0.01), however there was no difference between testosterone levels at 30 and 60 minutes (p = 1.0). The entire cohort had a decrease of approximately 18.6 ± 3.1%, 60 minutes after ingestion of the glucose and protein beverage, independent of the pubertal stage (Fig. 4.2). A decrease in LH of 0.4 ± 0.1 IU/L 60 minutes after the ingestion of the beverage was also seen (p < 0.01). The decrease in LH level was significantly correlated to the decrease seen in testosterone level (Fig. 4.3) (r = 0.52; p = 0.02).

For all subjects, consumption of the beverage resulted in higher insulin and GLP-1, and lower LH, FSH, testosterone, and ghrelin (Table 4.1). The change in testosterone was not significantly correlated with either the AUC for ghrelin (r = .412, p = 0.06) or GLP-1 (r = .17, p = 0.49). Changes in testosterone level had no significant relationship with percentage
body fat (r = 0.64, p = 0.08), WBISI (r = 0.005, p = 0.98), or the mean of the combined appetite scores (r = -0.16, p = 0.48).

4.5 Discussion

This is the first study to demonstrate that enteral glucose/protein intake acutely lowers testosterone levels in overweight and obese adolescent boys across puberty. Furthermore, it shows that the postprandial decreases of testosterone levels may be centrally mediated as indicated by lowering of LH levels after the beverage. The ~19% decrease of testosterone from glucose/protein intake in these boys is consistent with a 25% lower testosterone levels in adults males after a glucose beverage [10]. Not surprisingly, mid-late pubertal boys had greater absolute decreases in testosterone after the beverage in comparison to early pubertal boys however the relative change in testosterone was similar between the two. Fasting testosterone was inversely related to adiposity and ghrelin but not to insulin sensitivity. Change in testosterone in response to the beverage was significantly related to change in LH, but not correlated to adiposity, insulin sensitivity nor to changes in ghrelin or GLP-1.

The decrease of both LH and testosterone levels following enteral glucose/protein intake may be age-related and more pronounced in children. A study of older men (mean age 51 ± 1.4 years) undergoing an OGTT found a lowering of testosterone [10] but not LH, however, a study of adult men across a wider age range found that the testosterone decrease was related to lowered LH pulsatility, and that these effects were more pronounced in younger men [367]. Therefore, the decrease in testosterone in peripubertal children after glucose/protein intake may be centrally mediated via lowering of LH levels. Consistent with this possibility, a previous study in adult males demonstrated that changes in LH concentrations correlate with changes in serum testosterone levels within 40-120 minutes, in line with our measurements [368]. Although it would be expected that the decrease in LH would occur prior to testosterone if the testosterone decrease was centrally mediated, our study showed a concomitant decrease of LH and testosterone at 15 minutes. It may be possible that the LH decrease preceded the decrease in testosterone between baseline and 15 minutes, however earlier samples were not available for analysis.

While we show that the mixed beverage reduced testosterone, it is not clear if the protein or the glucose component was the primary stimulant for this change. The response observed may be primarily due to the glucose content of the beverage. Previous work has shown that
increased glucose uptake into the central nervous system via glial GLUT-1 receptors in the hypothalamus [369] and glucose stimulation of the intestinal-mucosal vagal pathway feeding back to the central nervous system results in lower LH [327, 370].

As postprandial GLP-1 increases have been proposed to explain the decrease of testosterone after glucose intake [327], this study explored the association between GLP-1 and testosterone. GLP-1 response to the beverage was unrelated to either fasting testosterone or its response and contrasts with the effect of GLP-1 infusion during a glucose challenge which decreased testosterone pulsatility in adult males, implicating a down-regulatory effect of GLP-1 on testosterone [327]. Because GLP-1 is more strongly stimulated by whey protein than glucose beverages [371] it is interesting that, while the protein/glucose beverage increased both GLP-1 and insulin, we did not detect a relationship between GLP-1 and testosterone change. It may be possible that this is a consequence of methodological differences as GLP-1 stimulation in our study occurred via enteral beverage intake rather than intravenous delivery as used in the previous study. Furthermore, the study by Jeibmann et al. [327] found decreases of testosterone pulsatility but not mean testosterone levels after the GLP-1 infusion.

Although fasting ghrelin and fasting testosterone were inversely correlated, there was no association between the change in ghrelin and testosterone after ingestion of the beverage. These results in the boys after an overnight fast are consistent reports in animals [320] and peripubertal boys [372]. In addition, higher ghrelin is associated with reduced spermatogenesis and smaller Leydig cell size in animals and humans [373]. Furthermore, ghrelin is typically lower in overweight/obese adolescents than normal weight adolescents [356] and in overweight subjects, post-meal ghrelin declines more slowly than in lean subjects [374], though our study did not immediately establish a direct association with postprandial ghrelin and testosterone.

The interpretation of these results has several limitations. Although a statistically significant decrease was seen in testosterone levels, it does not necessarily imply a biological significance. Both adult males and pubertal boys exhibit a diurnal rhythm in testosterone, with highest levels early morning, declining gradually to a nadir in the evening, so it is not possible to determine how much of the decrease in testosterone seen was related to this phenomenon, nor how long the effect may have lasted. Studies in adult males, however, do indicate a decline in testosterone with a subsequent rise at 120-150 minutes following a
glucose drink, indicating that the testosterone changes were at least, in part, due to nutrient intake, and not due to diurnal rhythm alone [327, 365]. It was not possible to separate the role of glucose and protein contained within the beverage on the depression of testosterone in this study, and unfortunately, blood samples were not available to test for changes in testosterone from the oral glucose tolerance test conducted earlier. We did not assess other macronutrients, in particular fat, which has been examined in adults studies that demonstrate plant-based, but not animal-based fat in mixed meals results in lowering of testosterone [375].

4.6 Conclusions

Our findings suggest that acute glucose-protein intake lowers testosterone levels in overweight and obese males across pubertal stages and that this effect may be mediated by a reduction in LH. However, it is not known if these acute changes in testosterone may themselves impact on appetite and short-term feeding behaviours. Further studies to explore the acute and chronic ingestion of glucose, protein and fat, and their individual effects on LH and testosterone in obese boys and adolescents are warranted.
Table 4.1 - Subject Characteristics and change in hormone levels following the glucose/protein beverage (n= 23). Subjects are also presented in pre-early puberty (n= 10) and mid-late puberty (n= 13).

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>60 min. post-drink</th>
<th>Baseline</th>
<th>60 min. post-drink</th>
<th>Baseline</th>
<th>60 min. post-drink</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>13.3 ± 2.6</td>
<td>11.2 ± 0.6</td>
<td>14.8 ± 0.7</td>
<td>4.7 ± 0.3</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td>161.4 ± 12.0</td>
<td>154.3 ± 3.4</td>
<td>166.8 ± 3.3</td>
<td>4.7 ± 0.3</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>89.2 ± 31.8</td>
<td>69.3 ± 5.0</td>
<td>104.4 ± 10.5</td>
<td>2.4 ± 2.5</td>
<td>2.3 ± 1.0</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td><strong>FM (kg)</strong></td>
<td>37.6 ± 19.9</td>
<td>29.7 ± 3.2</td>
<td>42.4 ± 7.4</td>
<td>4.7 ± 0.3</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td><strong>FFM (kg)</strong></td>
<td>54.6 ± 16.1</td>
<td>42.5 ± 3.4</td>
<td>62.0 ± 4.7</td>
<td>4.7 ± 0.3</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td><strong>Body Fat (%)</strong></td>
<td>39.5 ± 9.2</td>
<td>40.7 ± 2.6</td>
<td>38.8 ± 3.3</td>
<td>4.7 ± 0.3</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>33.1 ± 10.7</td>
<td>27.6 ± 1.4</td>
<td>37.3 ± 3.9</td>
<td>4.7 ± 0.3</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td><strong>BMI Z Score</strong></td>
<td>2.4 ± 0.4</td>
<td>2.3 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>4.7 ± 0.3</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td><strong>Glucose (mmol/L)</strong></td>
<td>4.7 ± 0.3</td>
<td>4.7 ± 0.7</td>
<td>4.7 ± 0.2</td>
<td>4.7 ± 0.3</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td><strong>Insulin (pmol/L)</strong></td>
<td>121.4 ± 93.5</td>
<td>477.8 ± 312.0 **</td>
<td>123.8 ± 34.4</td>
<td>447.9 ± 93.3 **</td>
<td>119.2 ± 33.7</td>
<td>504.4 ± 137.3 **</td>
</tr>
<tr>
<td><strong>WBISI</strong></td>
<td>3.4 ± 2.5</td>
<td>3.3 ± 1.0</td>
<td>3.4 ± 0.8</td>
<td>4.7 ± 0.3</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td><strong>LH (IU/L)</strong></td>
<td>1.7 ± 1.1</td>
<td>1.3 ± 0.9 **</td>
<td>0.9 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>2.1 ± 0.3 **</td>
</tr>
<tr>
<td><strong>FSH (IU/L)</strong></td>
<td>2.1 ± 0.9</td>
<td>1.8 ± 0.8 **</td>
<td>1.7 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>2.1 ± 0.3 **</td>
</tr>
<tr>
<td><strong>Testosterone</strong></td>
<td>6.8 ± 6.0</td>
<td>5.5 ± 4.8 **</td>
<td>2.3 ± 0.7</td>
<td>1.8 ± 0.5*</td>
<td>9.9 ± 1.9</td>
<td>7.7 ± 1.5 **</td>
</tr>
<tr>
<td><strong>nmol/L</strong></td>
<td>3.7 ± 2.7</td>
<td>5.3 ± 3.9 *</td>
<td>4.6 ± 1.7</td>
<td>7.5 ± 2.2*</td>
<td>4.7 ± 1.1</td>
<td>5.0 ± 1.1</td>
</tr>
</tbody>
</table>

* denotes significant difference from baseline at p < 0.05 and ** denotes significant difference from baseline at p < 0.01.
Figure 4.1- Association of fasting active ghrelin and fasting testosterone in males at all pubertal stages (n= 19).
Figure 4.2 - Testosterone level changes from baseline to 60 minutes after ingesting the glucose/protein beverage in pre-early puberty (n = 8) and mid-late puberty (n = 13). Data are means ± (SEM); N = 10-13/group. Treatment (beverage): p<0.0001; pubertal stage: p<0.001; treatment x pubertal stage: p < 0.005. * p<0.05, post-beverage at 60 minutes vs. baseline. ‡ p<0.05 between pre-early puberty and mid-late puberty.
Figure 4.3- Association of changes in testosterone and luteinizing hormone levels in boys following a glucose/protein beverage (n = 21).
Chapter 5

ACUTE DECREASE IN SERUM TESTOSTERONE AND APPETITE AFTER GLUCOSE AND PROTEIN BEVERAGES IN ADOLESCENT MALES

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ACUTE DECREASE IN PLASMA TESTOSTERONE AND APPETITE AFTER EITHER GLUCOSE OR PROTEIN BEVERAGES IN ADOLESCENT MALES

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5.1 Abstract

Objective: Chronic testosterone blood concentrations associate with food intake (FI), but acute effects of testosterone on appetite and effect of protein and glucose consumption on testosterone response has had little examination.

Methods: In a randomized, crossover study, twenty-three adolescent (12-18 y old) males were given beverages containing either: 1) whey protein (1g/kg bodyweight), 2) glucose (1g/kg bodyweight) or 3) a calorie-free control (C). Plasma testosterone, luteinizing hormone (LH), GLP-1 (active), ghrelin (acylated), glucose, insulin and subjective appetite were measured prior (0) and at 20, 35 and 65 minutes after the consumption of the beverage. FI at an ad libitum pizza meal was assessed at 85 min.

Results: Testosterone decreased acutely to 20 min after both protein and glucose with the decrease continuing after protein but not glucose to 65 minutes (\( p = 0.0382 \)). LH was also decreased by both protein and glucose but glucose had no effect at 20 min in contrast to protein (\( p < 0.001 \)). Plasma testosterone concentration correlated positively with LH (\( r = 0.58762, p < 0.0001 \)) and negatively with GLP-1 (\( r = -0.50656, p = 0.0003 \)). No associations with appetite, ghrelin or glycaemic markers were found. Food intake was not affected by treatments.

Conclusion: Protein or glucose ingestion results in acute decreases in both plasma testosterone and LH in adolescent males. The physiological significance of this response remains to be determined as no support for testosterone’s role in acute regulation of food intake was found.

5.2 Introduction

Chronic blood testosterone concentrations have been associated with FI. The relationships between testosterone and growth velocity [8], fat-free mass (FFM), fat oxidation [376] and, indirectly, bone growth [133] in pubertal males have been well established. In adults, elevated circulating testosterone levels are associated with reduced risk of eating disorders during and after puberty [377] and salivary testosterone with greater taste preference for chili peppers [378] implying a relationship between testosterone and FI. Furthermore, advances in
pubertal status associate with increases in FFM and FI in boys [329]; short-term FI in 8-17-year-old males is higher than in females of similar body composition, height and weight status [264].

Animal models provide more direct evidence that testosterone affects FI. Androgen receptors are located in FI regulatory pathways in the hypothalamic-pituitary-gonadal axis [330]. Testosterone injections to pregnant rats affects development of the hypothalamic-pituitary orexinergic system, specifically the G protein-coupled orexin receptors 1 and 2 during the neonatal period [379]. Orchiectomy decreases FI in rodents [331, 332], an effect which is reversed by providing doses of testosterone to match normal physiological levels [331, 332]. Testosterone injections also have a stimulatory effect on FI in intact adult rats [333].

However, there has been little exploration of the effects of FI ingestion on acute responses in testosterone concentrations or the acute effects of testosterone on FI, appetite or appetite regulatory hormones in humans. Ingestion of glucose by adult men [10] or a mixed drink of glucose and protein by adolescent males [380], decrease testosterone concentrations. In adult males, a rapid 25% decrease and suppression of testosterone levels over two hours was found after a 75g oral glucose tolerance test [10]. Similarly, ingestion of a mixed glucose-protein (60 g) beverage resulted in an 18% decrease of plasma testosterone levels after only one hour in overweight/obese adolescent males [372]. Neither of these studies measured the relationship of plasma testosterone with subjective appetite and food intake. However, the possibility is suggested by the observation that testosterone administration to peri-pubertal boys aged 8-12.5 years lowered circulating levels of the appetite-related hormones ghrelin and leptin [372].

Therefore, the hypothesis of this study was that the macronutrient composition of food affects acute post-ingestion responses in testosterone which in turn associates with post-ingestion responses in FI, appetite and appetite hormones in adolescent boys. The objective of this study was to compare the effect of glucose or protein provided at 1g/kg body weight in a beverage form on acute responses of plasma testosterone, LH, glucose, insulin, GLP-1, ghrelin, appetite, and FI.
5.3 Materials and Methods

5.3.1 Subjects

Normal weight and overweight/obese adolescent males (n = 23) 12 to 18 y of age were recruited via print advertisement in a local Toronto newspaper. After an initial phone contact, participants were scheduled for a screening session to determine their eligibility for participation in a study. Overweight was categorized as in the 85th-95th percentile and obesity in ≥95th percentile [21]. Participants’ height (m) and weight (kg) were measured using a stadiometer and a digital scale, respectively. Age-specific BMI percentiles were calculated using WHO growth charts [17]. Body composition was estimated with bioelectrical impedance analysis (RJL Systems BIA, 101Q) using the Horlick equation [381]. During the first experimental visit, a nurse trained by a pediatric endocrinologist examined all participants to assess their pubertal status using the Tanner method [59]. Participants were excluded from study if they had a history of prematurity, chronic illness or were taking any medications known to affect glucose homeostasis, appetite or pubertal development. Twenty-four 12-18 y old males completed the study, but only adolescent males in mid-late puberty with testosterone values above 150 ng/dl (which is the lower range value for what is expected of males in mid-late puberty [215]), were included in this analysis (n=23). All study participants were familiarized with the visual analog scales (VAS) used in the experimental sessions, and informed written consent was obtained from them and their legal guardians.

The study was approved by the University of Toronto Research and Ethics Board for Humans.

5.3.2 Protocol

After a 12 h fast, participants arrived at 08:30 am and were administered a motivation-to-eat VAS, which measures dimensions of subjective appetite and has been previously validated for use in boys after glucose and whey protein preloads [281]. Protein and glucose beverages were chosen as previous research indicates that a mixed beverage in adolescent males results in decreased testosterone [380], but the independent contribution of each remains unclear. The 100 mm scale was composed of four questions anchored at each end with contrasting
terms: (1) How strong is your desire to eat? (desire to eat, “very weak” to “very strong”), (2) How hungry do you feel? (hunger, “not hungry at all” to “as hungry as I’ve ever felt”), (3) How full do you feel? (fullness, “not full at all” to “very full”), and (4) How much food do you think you can eat? (prospective food consumption or PFC, “nothing at all” to “a large amount”). Participants were instructed to read each question and place an “x” along the 100 mm line depending on their current feelings. An aggregate score of these four questions was determined as the mean subjective appetite score as previously described in our laboratory [382-384].

Following the completion of the questionnaires, an intravenous catheter was placed for blood sampling. The first blood draw was taken prior to ingesting the experimental beverage at baseline (0 minutes). Participants were then given 5 minutes to ingest the beverage, and blood was later obtained 20, 35 and 65 minutes after baseline blood draw. Plasma was separated and stored at -80°C for later measurement of testosterone, glucose, insulin, glucagon-like peptide-1(GLP-1, active), active ghrelin, and luteinizing hormone (LH). The pizza meal was provided at 65 minutes as it was 60 minutes after completion of the drink (Fig. 5.1), when a post-glucose load nadir for testosterone is reached in adults [332] and following a mixed protein and glucose beverage in adolescent males [380].

The experimental beverages contained either 1g of glucose monohydrate (BioShop Canada Inc., Burlington, Ontario, Canada) or 1g of protein (plain whey-protein isolate; BiPro USA., Eden Prairie, Minnesota, U.S.A) per kg of bodyweight. Independent third-party analysis (Maxxam Analytics, Mississauga, Ontario, Canada) of the protein demonstrated 90.4% of the powder was protein, with 5.7% moisture, 2.2% ash, 1.18% fat, and 0.6% carbohydrates. Given these percentages, the protein beverage was 3.74 kcal/kg bodyweight while the glucose monohydrate beverage was 4 kcal/kg bodyweight. A non-calorie drink was used as control. All beverages were flavoured with 1.5ml of chocolate extract (Vanilla Food Company, Markham, Ontario, Canada) to account for the flavour differences and mixed with 500ml of water, similar to previous protocols [281]. The whey protein and control beverages were sweetened with 0.2g sucralose (Tate & Lyle, Stoney Creek, Ontario, Canada) in order to match sweetness with the glucose beverage. Sucralose was chosen as it has been shown to have no effect on postprandial plasma glucose or insulin [385]. Test beverages were prepared the evening before the study and refrigerated in order to be served chilled the following morning. Participants were served the drink in a large covered opaque cup through a straw.
The beverage was consumed within 5 min, followed by 50 mL of water to minimize aftertaste.

After the final blood draw, an *ad libitum* pizza meal was provided. The participants were instructed to eat during the next 20 min until they were comfortably full. Based on the participant preferences determined during screening, two varieties of Deep ‘N Delicious 5-inch-diameter pizza were provided for consumption; pepperoni or three-cheese pizzas (McCain Canada Ltd., Florenceville, Ontario, Canada). Pepperoni pizza (87g) contained 9g of protein, 6g of fat and 23g of carbohydrates for a total energy content of 180 kcal. Each three-cheese pizza (81g) contained 10g of protein, 6g of fat and 23g of carbohydrate for a total energy content of 180kcal. The cooked pizzas were weighed and cut into four equal pieces before serving, and the amount left after the meal was subtracted from the initial weight and converted to kilocalories to provide a measure of FI. These pizzas lack an outer crust resulting in a uniform energy content thus mitigating the opportunity for the participants to eat the energy-dense filling and leave the outside crust of the pizza. The additional water consumption was determined by weight.

5.3.3 Biochemical Assays

*Analysis of testosterone in plasma using gas chromatography-mass spectrometry*

Plasma concentrations of total testosterone were measured by gas chromatography-mass spectrometry (GC-MS) using a modified stepwise procedure for electron capture negative chemical ionization adapted from the methods of Fitzgerald, Griffin, and Herold appropriate for both plasma and serum samples [181]. Briefly, 2 ng of 16,16,17-d₃ testosterone (Sigma-Aldrich, St. Louis, MO, USA #T5536-1ML) internal standard was added to 250 µL of plasma. 2mL of ethyl acetate was added to each sample and samples were then vortexed for 2 min each followed by centrifugation at 1,850 g for 10 min. The organic layer was then transferred into clean tubes and evaporated under nitrogen at 40°C. Pentafluorobenzylxoxime derivatization were done by adding 50 µL of pyridine and 50 µL of Florox reagent and heated at 70°C for 1 h. Following which, samples were evaporated under nitrogen at 40°C prior to trimethylsilylation with BSTFA. Samples were then evaporated under nitrogen at 40°C and resuspended in 50 µL of heptane for analysis by GC-MS. GC-MS analysis of testosterone was carried out on an Agilent 5977A single-quadruple mass spectrometer coupled to an
Agilent 7890B gas chromatograph (Agilent Technologies, Mississauga, ON) in negative chemical ionization mode. Derivatized samples were injected via an Agilent 7693 autosampler (Agilent Technologies) into a DB-1 15 m x 0.32 mm (i.d.) x 0.25 µm film thickness capillary column (J&W Scientific from Agilent Technologies #123-1012) interfaced directly into the ion source. Injector port and interface temperatures were held at 260°C and 280°C, respectively. The oven temperature was programmed to 160°C for a hold time of 1 min, followed by a ramp to 280°C at 20°C per minute for a hold time of 3 min, followed by a ramp to 315°C at 25°C per minute for a hold time of 4 min. The final ramp and temperature hold was programmed to remove sterol contaminants from the capillary column between runs. The inlet was operated in splitless mode and helium carrier gas flow rate was constant at 1.8 mL per min. The reagent gas for negative chemical ionization was methane (99.999% purity) with a gas flow of 40%. The mass spectrometer was operated in select ion monitoring, scanning for \( m/z \) 535.2 ± 0.5 and \( m/z \) 538.2 ± 0.05 corresponding to testosterone and 16,16,17-d₃ testosterone, respectively. Dwell times for each ion were set to 0.1 sec. The mass spectrometer was calibrated daily and a gain factor of 3.33 was applied. Testosterone was quantified against a 4-point standard (200ng/dl, 400ng/dl, 800ng/dl and 1200ng/dl) curve prepared daily. The lower limit for detection of testosterone with this method is 5 ng/dl and the interassay coefficient of variation (inter-CV) is <5.8%.

**Biochemical assays**

Concentration of glucose in the plasma was measured using a glucose oxidase kit (Teco Diagnostics, Anaheim, CA, USA; intra-CV: <4.8%; inter-CV: <4.3%; Cat. # G519-1L). Insulin was measured using the enzyme-linked immunosorbent assay (ELISA) (ALPCO, Salem, NH, USA; intra-CV: <10.3%; inter-CV: <16.6%; Cat. # 80-INSHU-E10.1). LH was also assayed with ELISA (Diagnostics Biochem Canada, London, ON, Canada; intra-CV: <4.5%; inter-CV: <9.2%; Cat. # 80-CAN-LH-4040). Human active GLP-1 (intra-CV: <8%; inter-CV: <12.8%; Cat. # EGLP-35K) and acylated ghrelin referred to herein as active ghrelin (intra-CV: <7.5%; inter-CV: <12.9%; #EZGRA-88K) were also measured with ELISA kits (Millipore, Billerica, MA, USA).
5.3.4 Statistical Analyses

Sample size required to detect a treatment effect on testosterone was assessed using PS Power and Sample Size Calculations (Version 3.0.43, 2009, Vanderbilt University). The data from three studies in adult males showed decreases of testosterone of 24% following an OGTT [10, 365, 366]. Based on these data, and by using mean and standard deviation values from a study that measured testosterone in boys from T1 to T5 puberty [208], 13 boys were required to achieve power of 0.8 at $\alpha = 0.05$. A similar sample size was estimated for FI, based on a previous study from our laboratory [281] showing effects on caloric intake at a meal 60 min later when similar glucose and protein beverages were provided to normal and overweight obese boys. The usual requirements of probability of Type-1 error ($\alpha$) of 0.05 and Type-2 error ($\beta$) of 0.1, i.e., power =0.8, indicated a sample size of 24 participants was required. However, because a sample size of 22 is required for measures of subjective appetite and FI [386], a total of 23 participants were recruited.

Statistical analyses were conducted using SAS version 9.4 (SAS Institute Inc, Cary, NC, USA). For analyses of all measurements, the baseline value was subtracted from postprandial responses to account for between-subject differences. All data were tested for normality. Due to the diurnal variation of sex hormones in adolescent males, statistical analysis and presentation of the results are based on change from baseline. Analysis of covariance (ANCOVA) of the outcome with the baseline as covariate, and analysis of variance (ANOVA) of change from baseline are both current and unbiased methods of analysis employed in randomized studies [387]. ANCOVA is often preferred as it has more power, but it also assumes absence of a baseline difference. Because the protein group differed significantly from control ($p=0.0293$) and from glucose ($p=0.0426$) at pretest (baseline) for luteinizing hormone, in order to minimize bias, ANOVA of change from baseline was chosen as preferred statistical test. Differences in sex hormones (testosterone, LH), gastrointestinal hormones (GLP-1, ghrelin), glycaemic markers (blood glucose and insulin) and appetite scores changes from baseline were examined using a two-way repeated measures analysis of variance via PROC MIXED procedure (PROC GLIMMIX for non-normal data) followed by Tukey-Kramer post-hoc test to analyze the main effects of treatment, weight and time and its interactions. When a treatment by time interaction was found, one factor ANOVA was used followed by Tukey-Kramer post-hoc test to compare the effect of treatments at each time of measurement. Results were pooled for normal weight and overweight adolescents, unless a
significant weight effect was found. Food intake was analyzed by one-factor ANOVA. Pearson correlation coefficients (for normal data) and Spearman’s rank coefficients (for non-normal data), were calculated via PROC CORR to detect the associations between testosterone and LH with appetite scores, GLP-1, ghrelin, blood glucose and insulin. All results are presented as mean±standard error of the mean (S.E.M.). Statistical significance was determined at $p < 0.05$.

### 5.4 Results

Participant characteristics are presented in **Table 5.1**. A total of 23 adolescent males age 12 to 18 that were classified in the mid-late pubertal stages completed the study and were included in the subsequent analysis. Eleven were overweight/obese and were approximately 50% higher in body weight with a three-fold higher fat mass but similar FFM compared to the 12 normal weight participants.

Average baseline levels of appetite- and sex-related hormones are shown in **Table 5.2**. Overweight/obese participants had higher fasting blood glucose and insulin concentration than the normal weight participants and lower baseline testosterone levels.

**Effects of treatments over time on responses in hormones and glucose concentrations**

**Sex hormones**

Plasma testosterone concentrations were affected by treatment ($F = 3.59, p = 0.0382$) and weight status ($F = 5.26, p = 0.0322$), but not by time ($F = 1.49, p = 0.2365$). An interaction was found between weight status×treatment ($F = 7.38, p = 0.0021$). No interactions were found between treatment×time ($F = 0.78, p = 0.5428$), weight status×time ($F = 0.55, p = 0.5812$), or treatment×time×weight status ($F = 1.34, p = 0.2654$) (**Figure 1**). Testosterone concentrations were decreased from baseline over time. The decrease in testosterone after protein ingestion was prolonged to 65 min. However, the peak response after glucose occurred at 20 min and was sustained at that concentration to 65 min.

Overall, normal weight subjects had a greater decrease of testosterone when compared to overweight subjects (-153.31 ± 30.35 ng/dl vs. -51.39 ± 32.44 ng/dl, $p = 0.0322$) (**Figure 5.2** B). The significant interaction between treatment and weight status was attributed to the
response (presented as the change from baseline, Figure 5.2 A) in the control as well as the response to whey and glucose. In the overweight subjects, testosterone response to both protein and glucose treatments were different from control (p=0.0205, p=0.0022 respectively); however, testosterone response to glucose and protein were not different (p=0.6223). For normal weight subjects, there were no differences of testosterone response between any of the treatments (p >0.05).

Plasma LH was affected by treatment (F = 21.37, p <0.0001) but not time (F = 1.26, p = 0.2943) or interaction (F = 1.23, p =0.3051) (Figure 5.3). Both glucose (p = 0.0195) and protein (p < 0.0001) treatments reduced LH when compared with control, but more after protein than after glucose (p = 0.0016). The response to protein, but not glucose was apparent at 20 min.

**Glucose, Insulin, GLP-1, and Ghrelin**

Plasma glucose concentrations were affected by treatment (F = 118.70, p<0.0001), time (F = 5.08, p = 0.0107) and a time×treatment interaction (F = 4.54, p = 0.0035) (Figure 5.4). Post-treatment plasma glucose concentrations were higher at all times after the glucose treatment when compared to both protein and control beverages (p < 0.05) with no differences between protein and control treatments.

Plasma insulin (Figure 5.5) was affected by time (F = 6.49, p = 0.0036), treatment (F = 115.96, p < 0.0001) and a time×treatment interaction (F = 4.67, p = 0.002). Post-treatment plasma insulin concentration was higher after glucose, than after protein and higher after both than control all time points (p < 0.05).

GLP-1 was affected by treatment (F = 18.26, p < 0.0001) but not by time (F = 1.48, p = 0.24) (Figure 5.6). There were no significant interactions between treatment×time (F = 2.09, p = 0.09). GLP-1 (active) average concentrations were similarly increased by both the glucose and protein beverages when compared to the control beverage (p = 0.0003, p <0.0001, respectively). Glucose and protein were not different (p = 0.2495).

There was an effect of treatment (F = 29.72, p < 0.0001) on mean active ghrelin, with no effect of time (F= 0.37, p = 0.69) and no interactions of treatment×time (F = 1.23, p < 0.3) (Figure 5.7). Ghrelin (active) was decreased by glucose (p < 0.001) and protein (p < 0.001)
when compared to control with no difference between glucose and protein treatment \((p = 1308)\).

**Appetite**

There was an effect of time \((F = 9.05, p = 0.0005)\) and treatment \((F = 5.09, p = 0.0103)\) on appetite (**Figure 5.8**) with no treatment×time interaction \((F = 0.26, p = 0.904)\). The glucose beverage, but not protein, lowered post-treatment subjective appetite when compared with the control \((p = 0.0198)\). Additionally, glucose treatment appetite score was lower than after the protein treatment \((p = 0.0247)\).

**Food Intake**

There was no effect of the treatment \((F = 1.79, p = 0.782)\) or weight status \((F = 2.23, p = 0.14)\) on total caloric intake. Mean FI for the control, glucose and protein treatments were 1409.93 ± 97.7 kcal, 1280.18 ± 95.2 kcal and, 1325.18 ± 95.9 kcal, respectively.

**Correlations**

Post-treatment decrease of testosterone was correlated with decreased LH \((r = 0.27, p = 0.0050)\) and was inversely correlated to increases in GLP-1 \((r = -0.39, p = 0.0004)\). Lower post-treatment testosterone concentrations over time did not correlate with ghrelin \((r = 0.20, p = 0.1531)\), glucose \((r = 0.25, p = 0.1398)\), or insulin \((r = -0.22, p = 0.1455)\) (**Table 5.3**). Testosterone decrease was not correlated to average appetite \((r = 0.05, p = 0.7456)\). Neither was testosterone change from baseline before the meal with FI \((r = 0.14, p = 0.2800)\). LH was not correlated to any other parameter but testosterone (**Table 5.3**).
5.5 Discussion

The results of this study support our hypothesis that macronutrient composition of food affects the acute post-ingestion response of testosterone in adolescent boys. However, they provide no support for the hypothesis that these responses contribute to regulation of appetite or FI.

The testosterone decrease in response to protein and glucose ingestion is consistent with previous research. A decrease in testosterone has been reported in adult males after glucose ingestion [10] and in overweight adolescent boys given a combined protein/glucose beverage [380]. The novel contribution of this study is that it shows that whey protein suppresses testosterone, and may have a longer duration of effect than glucose suppression, indicating that the effects of food ingestion are not only explained by energy sensing. Further support for a greater role of protein over carbohydrate ingestion and its greater duration of effect arises from observations of lower fasting testosterone concentrations in adult males following high protein (low carbohydrate) diets [334, 335]. The slight but statistically insignificant change of testosterone in the control treatment is concordant with normal diurnal rhythms of testosterone in adolescent males who typically display early morning peak testosterone and late evening nadir [203]. Furthermore, obesity has been shown to alter diurnal patterns of testosterone in males and lower morning testosterone values [388]. Though both groups had elevated LH levels during the control treatment, overweight subjects had significantly lower testosterone at baseline. It is therefore possible that overweight boys are more responsive to increased LH, however this cannot be concluded from these results.

The decrease of testosterone may be independent of LH and initiated by the intake of glucose or amino acids, particularly leucine, which stimulates rapamycin (mTOR) signaling and subsequent protein synthesis [389]. Leucine and glucose also inhibit adenosine monophosphate-activated protein kinase (AMPK) [390, 391], which is upstream of mTOR and prevents protein biosynthesis [392] in addition to inhibiting androgen receptor (AR) mRNA expression [393]. Inhibition of AMPK through glucose or protein intake may increase AR expression leading to greater testosterone uptake by the muscle tissue which would lower plasma testosterone levels.

No associations were found between the testosterone and appetite responses. Lower post treatment mean testosterone concentrations over time cannot be interpreted as support for any
effect of testosterone on appetite. Pre-meal testosterone concentrations were not correlated to appetite or FI. However, the appetite responses are consistent with the known effects of carbohydrate ingestion on short term appetite [382], but contrast to other reports that protein increases satiety in both adults [383] and adolescents [281]. In overweight and obese boys, appetite decreases after both protein and glucose ingestion [357], but glucose suppresses FI more than whey protein whereas normal weight boys respond equally to both [281].

The lack of effect of the treatments on FI is surprising because the doses were adjusted for body weight to accommodate the range of bodyweights of the participants. In a previous study, protein and glucose beverages provided at 1g/kg body weight to 12 normal and 12 overweight obese boys, averaging 12 y of age, reduced FI at a meal 60 min later by an average of 17% with a greater reduction after protein [281]. Although the different results of this and the prior study cannot be explained, the absence of effect of the treatments on FI also provides evidence that testosterone does not function in short-term FI regulation. Testosterone concentrations after protein were at their lowest 60 min immediately prior to the ad libitum pizza meal, yet no correlation was found between FI and testosterone at 60 min. It remains possible, however, that a relationship would be found by extending the measures of the testosterone and the time of the later meal.

Although the study provides no evidence that testosterone is a regulator of short term FI, the physiological effects of its acute decrease in response to the composition food merits further exploration. LH was measured because of its role in testosterone production. An interaction between treatment and time was indicated because LH increased over time in the control but decreased over time after protein and glucose (Figure 5.3). These responses were positively (r = 0.59, p < 0.05) associated with testosterone, consistent with the known interactions of these hormones. LH stimulates testosterone production via the Leydig cells of the testicles [74] and ghrelin suppresses the secretion of LH [394] and is present in Leydig cells [320].

Although few appetite hormones were measured, the pooled data show expected associations among them and with appetite. Conversely, none associated with testosterone, adding further doubt to any physiological significance of the association between testosterone and appetite. Their lack of association with FI is consistent with the literature showing little predictive strength in appetite hormone measures and overconsumption of foods in adolescent populations [267, 395].
Consistent with previous reports [9], overweight/obese boys had lower testosterone levels in comparison to the normal weight boys. Low testosterone concentrations in obese males result from aromatization of testosterone within adipose tissue [305, 311]. Weight status interacted with treatment effects on testosterone; normal weight subjects had a greater decrease in testosterone compared with overweight and obese boys. This may be due to lower baseline levels and insulin resistance. Insulin was higher at baseline with overweight/obese boys as is commonly observed in insulin resistant subjects [396]. This could in part be explained by obesity related insulin resistance which impedes insulin facilitated hypothalamic GnRH secretion, further depressing testosterone production in obese subjects [397, 398].

Due to the composition of the protein powder, there were minor caloric differences between the glucose and protein treatments; however, this difference is an unlikely factor in determining the results. The mean of the overweight subjects in this study was 88.5± 4.7 kg compared to normal weight 63.8± 3.5 kg resulting in treatment differences of only approximately 23 kcal for OW and 6 kcal for NW.

Further studies of the effect of quantity and protein source (rapidly vs. slowly digested) are also needed to explain mechanisms leading to reduction of plasma testosterone and the physiological significance of these acute effects of food. Additionally, the decrease in LH after protein or glucose ingestion is a novel observation, and further exploration of the acute effect of glucose and protein on sex hormone response in girls and women is merited.

5.6 Conclusions

Protein and glucose ingestion results in acute decreases in both plasma testosterone and LH in adolescent males. The physiological significance of this response remains to be determined as no support for a role of testosterone in acute regulation of food intake was found.
Table 5.1. Participant characteristics

<table>
<thead>
<tr>
<th></th>
<th>Normal Weight (n = 12)</th>
<th>Overweight (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>14.3 ± 0.4</td>
<td>15.6 ± 0.5</td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td>172.9 ± 2.7</td>
<td>171.8 ± 2.3</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>63.8 ± 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88.5 ± 4.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Fat-Free Mass (kg)</strong></td>
<td>53.6 ± 3.1</td>
<td>59.1 ± 1.8</td>
</tr>
<tr>
<td><strong>Fat Mass (kg)</strong></td>
<td>10.3 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.4 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Body Fat %</strong></td>
<td>16.0 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.3 ± 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>21.1 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.8 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>BMI %ile (WHO)</strong></td>
<td>67.5 ± 7.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.3 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>All values are means ± SEM, n = 23. Values in the same row with different superscript letters are significantly different, p < 0.05.

---

Table 5.2. Baseline levels of appetite- and sex-related hormones

<table>
<thead>
<tr>
<th></th>
<th>Normal Weight (n = 12)</th>
<th>Overweight (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Testosterone (ng/dl)</strong></td>
<td>1048.71 ± 103.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>765.49 ± 46.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>LH (IU/L)</strong></td>
<td>3.65 ± 0.30</td>
<td>3.46 ± 0.38</td>
</tr>
<tr>
<td><strong>Ghrelin, active (pg/ml)</strong></td>
<td>288.37 ± 33.54</td>
<td>174.33 ± 29.22</td>
</tr>
<tr>
<td><strong>GLP-1, active (pM)</strong></td>
<td>4.81 ± 0.37</td>
<td>7.93 ± 1.51</td>
</tr>
<tr>
<td><strong>Insulin (uIU/ml)</strong></td>
<td>6.77 ± 0.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.21 ± 1.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Glucose (mg/dl)</strong></td>
<td>90.69 ± 1.96</td>
<td>97.15 ± 1.85</td>
</tr>
</tbody>
</table>

Values were calculated by averaging the baseline levels of all three of the sessions for each marker. All values are means ± SEM, n = 23. Values in the same row with different superscript letters are significantly different, p < 0.05.
Table 5.3. Relationships between testosterone and luteinizing hormone with dependent measures (Δ from baseline means)\(^1\).

<table>
<thead>
<tr>
<th>Dependent Measure</th>
<th>Testosterone (ng/dl)</th>
<th>Luteinizing hormone (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appetite score (mm)</td>
<td>NS</td>
<td>NS*</td>
</tr>
<tr>
<td>Luteinizing hormone (IU/L)</td>
<td>( r = 0.27338 )</td>
<td>-</td>
</tr>
<tr>
<td>GLP-1 (pM)</td>
<td>( r = -0.38993 )</td>
<td>NS*</td>
</tr>
<tr>
<td>Ghrelin (pg/ml)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin (pg/ml)</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^1\)Correlation coefficients \((r)\) indicate statistically significant associations between dependent measures, \(p<0.05\). NS=nonsignificant.

*Indicates Pearson correlation was calculated, otherwise Spearman’s rank coefficient was determined.

Figure 5.1. Experimental protocol. Subjects arrived in the laboratory after a 10-12h fast and were randomly assigned one of three beverage conditions.
Figure 5.2. Effect of glucose and protein on plasma testosterone over time. Statistics were performed on change from baseline. Treatment: \( p = 0.0382 \), Weight Status: \( p = 0.0322 \), Weight status \( \times \) Treatment \( p = 0.0021 \), Time: \( p = 0.2365 \), TRT \( \times \) Time: \( p = 0.5428 \), by two-way repeated measures ANOVA. Different superscripts are significantly different by Tukey–Kramer post hoc test, \( p < 0.05 \). All values are means \( \pm \) S.E.M.s; Total \( n=22 \), Normal Weight \( n=12 \), Overweight \( n=11 \).

Note: Embedded panels represent differential effects of treatments by weight status (A) and overall effect of weight status on plasma testosterone (B).

Figure 5.3. Effect of glucose and protein on plasma luteinizing hormone. Statistics were performed on change from baseline. Treatment: \( p < 0.0001 \), Time: \( p = 0.2943 \), TRT \( \times \) Time: \( p = 0.3051 \), by one-way repeated measures ANOVA.
ANOVA. Different superscripts are significantly different by Tukey–Kramer post hoc test, $p<0.05$. All values are means±S.E.M.s; n=23.

**Figure 5.4.** Effect of glucose and protein on plasma glucose. Treatment: $p<0.0001$, Time: $p=0.0107$, TRT*Time: $p=0.0035$, by one-way repeated measures ANOVA. Different superscripts are significantly different by Tukey–Kramer post hoc test, $p<0.05$. All values are means±S.E.M.s; n=23.

**Figure 5.5.** Effect of glucose and protein on plasma insulin. Treatment: $p<0.0001$, Time: $p=0.0036$, TRT*Time: $p=0.0020$, by one-way repeated measures ANOVA. Different superscripts are significantly different by Tukey–Kramer post hoc test, $p<0.05$. All values are means±S.E.M.s; n=23.
Figure 5.6. Effect of glucose and protein on plasma GLP-1. Treatment: $p < 0.0001$, Time: $p = 0.2382$, TRT*Time: $p = 0.0909$, by one-way repeated measures ANOVA. Different superscripts are significantly different by Tukey–Kramer post hoc test, $p < 0.05$. All values are means±S.E.M.s; n=23.

Figure 5.7. Effect of glucose and protein on plasma ghrelin. Treatment: $p < 0.0001$, Time: $p = 0.6923$, TRT*Time: $p = 0.3042$, by one-way repeated measures ANOVA. Different superscripts are significantly different by Tukey–Kramer post hoc test, $p < 0.05$. All values are means±S.E.M.s; n=23.
Figure 5.8. Effect of glucose and protein on appetite score. Statistics were performed on change from baseline. Treatment: $p = 0.0103$, Time: $p = 0.0005$, TRT*Time: $p = 0.9042$, by one-way repeated measures ANOVA. Different superscripts are significantly different by Tukey–Kramer post hoc test, $p < 0.05$. All values are means±S.E.M.s; $n=23$. 
Chapter 6

ACUTE DECREASE IN APPETITE IS UNRELATED TO INCREASE IN PLASMA TESTOSTERONE DURING HIGH INTENSITY CYCLING IN ADOLESCENT MALES

The following chapter is a reproduction of a manuscript that will be submitted for publication to Applied Physiology, Nutrition and Metabolism
ACUTE DECREASE IN APPETITE IS UNRELATED TO INCREASE IN PLASMA TESTOSTERONE DURING HIGH INTENSITY CYCLING IN ADOLESCENT MALES

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6.1 Abstract

**Background and Objectives:** High intensity exercise (HIEX) suppresses appetite, but the mechanism is unknown. Appetite regulating hormones have been explored. However, the effect of aerobic HIEX on the increase in testosterone and its relationship with appetite and appetite hormones has yet to be described. Therefore, the effect of HIEX on testosterone levels and the relationship between testosterone responses, selected appetite hormone and appetite was explored in teenage males.

**Participants and Methods:** In a randomized, crossover study, fifteen adolescent boys age 16.3 ± 0.3 completed two sessions of either: 1) three 10-minute bouts of HIEX cycling at 75% VO₂peak or 2) remained at rest. Plasma testosterone, PYY, ghrelin, GLP-1 and subjective appetite were measured before (0 min) and after (60 min) HIEX.

**Results:** Plasma testosterone concentrations increased by 21.5 ± 13.6% after 60 min of HIEX when compared to rest (p = 0.0215). HIEX reduced ghrelin (p < 0.01), desire to eat (p = 0.026) and hunger (p = 0.022). However, testosterone was not correlated to appetite (r = -0.05, p = 0.87), ghrelin (r = -0.14, p = 0.67), GLP-1 (r = -0.02, p = 0.95 or PYY (r = 0.29, p = 0.41) responses during HIEX.

**Conclusion:** The increases in plasma testosterone levels after aerobic HIEX in adolescent males does not associate with the acute decrease in appetite or responses in appetite hormones after HIEX.
6.2 Introduction

In adult males, both anaerobic and aerobic modes of exercise, including weight lifting [337] or cycling [338] and running [339] increase plasma testosterone. High-intensity exercise (HIEX) reduces appetite and alters levels of appetite hormones [272, 399, 400] and inflammatory responses [401, 402]. However, the mechanism by which HIEX reduces appetite is unknown, but a role for testosterone can be suggested from several lines of evidence.

Elevated testosterone may be related to food intake (FI). It has been linked with modifying taste acuity [378] and a reduced risk of peri and post-pubertal eating disorders [377]. A role for testosterone in determining FI is indicated by observation that FI of pre-pubertal and adolescent boys is higher when compared to weight, height, and aged-matched females [264]. Animal studies support a role for testosterone and FI; Orchiectomy decreases FI in rodents [331, 332], but can be restored by testosterone injections to match normal physiological levels [331, 332]. Injections of testosterone also increase FI in adult rats with intact testicles [333].

Consumption of glucose or protein beverages are known to suppress appetite [281]. Recent studies also show that testosterone decreases immediately after adults consume a glucose beverage during an oral glucose tolerance test [10] and after children consume a mixed protein-carbohydrate beverage [380], or adolescents consume either protein or glucose beverages. In the latter, the decrease in testosterone was not associated with a decrease in appetite [403]. However, associations do not show cause and effect, and it has yet to be determined whether an increase of testosterone is associated with appetite in adolescent boys. HIEX, based on studies in adults, provides an alternative approach to providing direct injections of testosterone into healthy adolescents. In adolescents, HIEX decreases subjective appetite [401] however this does not typically reflect subsequent food intake [404, 405] and studies in populations with low or no testosterone such as obese [406] and women [407] show varied effects on appetite after HIEX.

As with adult males [337-339] adolescent males have demonstrated increased plasma testosterone after high intensity anaerobic exercise [344] but studies measuring the effect of aerobic exercise in this population [340, 346] failed to yield results due to methodological
shortcomings such as inadequate exercise intensity and poor sensitivity for detecting lower levels of testosterone in adolescents [408].

Therefore, the hypothesis of this preliminary study was that acute HIEX increases testosterone and decreases appetite in adolescent boys but the responses are unrelated. The objective of this study was to determine the effect of high-intensity repeated bouts of HIEX cycling on testosterone levels and appetite, and the relationship between these responses, in adolescent males aged 13 to 18.

6.3 Materials and Methods

6.3.1 Subjects

Subject recruitment was as previously described in detail [402]. Normal weight healthy adolescent males age 13 to 18 years were recruited via print ad in the Toronto Star newspaper.

A telephone questionnaire was employed to determine eligibility for this study. Weight status was defined using the Center for Disease Control BMI charts (BMI for age percentile: 15th-85th) [409]. Questions pertaining to physical activity readiness and eating habits were administered and scored. Exclusion criteria included fear of venipuncture, dieting history, and diabetes or other metabolic diseases. All experimental procedures have been approved by the University of Toronto Health Sciences Research Ethics Board, and informed consent was obtained from all participants, parents of the subjects as well as assent from the subjects themselves. Originally 21 participants were recruited through local advertisement; however, 6 participants did not complete the study, due to a mild form of vasovagal syncope, likely due to the IV-catheter insertion or due to difficulties scheduling the sessions.

6.3.2 Protocol

Design:

Blood samples for this secondary analysis were obtained from a study determining the effects of high-intensity exercise (HIEX) and anti-inflammatory medication on the relationship between inflammation and appetite [402]. In that study, 15 NW boys (aged 13-18y) were
randomly assigned in a crossover design to four treatments. Treatments were 1) Water+Rest; 2) Rest+Ibuprofen (IBU); 3) Water+HIEX; 4) HIEX+IBU. IBU (300mg) was provided in 250ml water. Data reported here are for the groups when exposed to the water at rest and water and HIEX treatments only. IBU groups were excluded due to the previously demonstrated inhibition of testicular testosterone production after acute IBU intake resulting in compensated hypogonadism [410].

HIEX consisted of three separate bouts of recumbent cycling between 60-70RPM at 75% VO_2Peak for 10 minutes per bout, similar to Thivel et al. [411]. Each exercise bout was followed by 1:30min of rest. Appetite ratings and plasma biomarkers of appetite, inflammation, stress and glucose control were measured. Upon arrival a catheter was inserted and at time 0 visual analogue scales were administered. At five minutes the participants drank water alone or with IBU. HIEX started at 30 min and terminated at 60 min (Figure 6.1). Blood was collected and visual analog scale questionnaires were completed at baseline (0 min) and 65 min after the start of each session.

**Participant Assessment**

The assessment and protocol were as previously described in detail [402]. Initial screening of participants was performed at the University of Toronto - Goldring Centre for High Performance Sports. Anthropometric measures such as age, height, body-weight, BMI, BMI for age percentile and percent body-fat were recorded and physical fitness was assessed via indirect calorimetry. Ventilatory gases were collected using a Moxus metabolic cart (AEI Technologies Inc., Pittsburgh, PA, USA), a facemask, and a 2-way non-rebreathing valve (Hans Rudolph, Inc., Shawnee, KS, USA). Inspiratory ventilation was measured with a pneumotachometer, the O2 and CO2 contents of mixed expired gas with an S-3A Oxygen Analyzer, and CO2 content with a CD-3A 251 Carbon Dioxide Analyzer (AEI Technologies Inc., Pittsburgh, PA, USA). Prior to each test, the metabolic cart was calibrated with known gas concentrations of 16.04% O2 and 4.06% CO2 and 20% O2 and 0.03% CO2. The Moxus metabolic cart has been validated over wide measurement ranges using two sensors for ventilation against the Douglas bag method [412].

The HIEX was conducted on a Kettler RE7 recumbent bicycle (Kettler, Ense-Parsit, NRW, Germany). The VET was assessed using the ventilatory equivalent method [413] and VO2peak determined using the highest six consecutive breaths [414]. Body composition was
estimated by bioelectrical impedance analysis (RJL Systems BIA, 101Q) using the Horlick equation [381]. All sessions were conducted at the University of Toronto Athletic Center on weekends between 9 and 10 am after a 12-h overnight fast.

**Visual Analog Scales**

Visual Analog Scales were provided to participants as previously described to evaluate subjective appetite [414-416]. Four questions measuring desire to eat, hunger, fullness and anticipated food intake were fixed at each end with contrasting terms on the 100 mm scale. Participants marked an “×” along the 100 mm line to reflect their feelings. The scales have been previously validated [281, 417].

### 6.3.3 Biochemical Assays

Blood samples for measurement of testosterone, GLP-1, ghrelin, and Peptide YY3-36 (PYY) were as described previously [402, 418]. Blood was collected into pre-chilled 10 mL BD Vacutainer™ (BD Diagnostics, Sparks, MD, USA) at baseline (0 min) and 65 min. Blood collection tubes contained spray dried K2EDTA anticoagulant, and a proprietary cocktail of protease inhibitors [e.g. DPP-IV (R-3-Amino-1-\([3-(trifluormethyl)-5,6,7,8-
 tetrahydro[1,2,4]triazol[4,3-a]pyrazin-7-yl]-4-(2,4,5-trifluorophenyl)butan-1-on), AEBSF (4-
(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride) and aprotinin (Trasylol)] to prevent the proteolytic breakdown of hormones. Immediately after collection, plasma was separated by centrifugation for 15 min at 2000 RCF at 4 °C, then aliquotted into 2 mL Eppendorf (Eppendorf, Hamburg, Germany). Furthermore, 200 µL 1 N HCl was added to every 1 ml of plasma collected for ghrelin analysis.

Human active GLP-1 (intra-CV: <8%; inter-CV: <5%; #EGLP-35K) and total and acylated ghrelin (intra-CV: <2%; inter-CV: <8%; #EZGRT-89K) were also measured with ELISA kits (Millipore, Billerica, MA, USA). Total PYY [i.e. PYY (1-36 amide) and PYY (3-36) was also measured using commercial ELISA kits purchased from Millipore (Millipore, Billerica, MA, USA) (intra-CV: <5.8%; inter-CV: <16.5%; #EZHPYYT66K). Samples were stored at -80°C until analysis, and all samples were run in duplicate.
**Analysis of testosterone in plasma using gas chromatography-mass spectrometry**

Plasma concentrations of total testosterone were measured as previously described in 5.3.3[418] by gas chromatography-mass spectrometry (GC-MS) adapted from Fitzgerald, Griffin, and Herold [181]. Testosterone was quantified against a 4-point standard (200ng/dl, 400ng/dl, 800ng/dl and 1200ng/dl) curve prepared daily. The lower limit for detection of testosterone with this method is 5 ng/dl and the interassay coefficient of variation (inter-CV) is <5.8%.

### 6.3.4 Statistical Analyses

Change in testosterone was used as the primary measure to calculate sample size. Sample size required to detect a treatment effect on testosterone was assessed using PS Power and Sample Size Calculations (Version 3.0.43, 2009, Vanderbilt University). Published data from the previous studies in our lab showed decreases of testosterone of 9-24% following glucose and protein treatments [380, 418]. Based on this data, 14 boys were required to achieve power of 0.8 at $\alpha = 0.05$. A similar sample size was estimated for measures of subjective appetite and has previously been validated to be reproducible for subjective appetite measures after exercise [386].

Paired sample t-tests were used to compare the absolute change of testosterone and other blood measures in addition to VAS between HIEX and rest. Non-parametric paired t-tests were used for non-normal data. Pearson correlation coefficients (for normal data) and Spearman’s rank coefficients (for non-normal data), were calculated via PROC CORR to evaluate testosterone compared to measures of VAS, ghrelin, PYY and GLP-1 as well as associations among all measures. All analyses were performed with Statistical Analysis Software, version 9.2 (SAS Institute Inc., Carey, NC, USA). The data are expressed as means ± SEM. Statistical significance was declared at $P < 0.05$. 
6.4 Results

A total of 15 adolescent boys age 16.3 ± 0.3 years old completed the study. Subjects were 174.8 ± 1.9 cm tall and weighed 65.4 ± 2.3kg with 23.4 ± 2.0% body fat and were considered normal weight under CDC BMI percentile (58.3 ± 5.1). Average VO2max values were 44.7 ± 1.2 ml/kg/min, which is considered slightly below the age average [419]. All participants had fasting testosterone values that were expected during mid-late puberty (>150ng/dl) [215] and no differences we detected at baseline between rest (868.16 ± 56.2 ng/dl) and HIEX (793.7 ± 50.8 ng/dl, p = 0.31).

Effects of exercise over time on responses in hormone and glucose concentrations

A. Testosterone.

Testosterone increased 21.5 ± 13.6% (126.99 ± 87.2 ng/dl) from baseline after 65 minutes of HIEX which was greater than when compared to testosterone change after 65 minutes of rest (-97.76 ± 90.4 ng/dl, p = 0.022) (Table 6.1). The mean testosterone increase from HIEX was significantly greater than the mean decrease observed during rest with a difference of 256.1 ± 90.1 ng/dl (p = 0.019) (Table 6.2).

B. Ghrelin, GLP-1, PYY, Glucose, Insulin,

Changes in plasma hormones are shown in Table 6.1. The decrease in ghrelin was greater after HIEX when compared with rest (p = 0.0017). There was no difference of change in GLP-1 (p = 0.81) or PYY (p = 0.46) between rest and HIEX.

Glucose was not affected by HIEX and there was no difference from rest values (p = 0.37). Though there was a trend of HIEX to decrease insulin, there was no difference of change in insulin between rest and HIEX (p = 0.73).

C. Appetite

Mean subjective appetite change was not affected by HIEX (-3.57 ± 8.2mm) when compared to rest (9.97 ± 5.1mm, p = 0.08). HIEX decreased the desire to eat (-2.13 ± 8.9mm) and hunger (-0.4 ± 7.3mm), which were different from the increases seen at rest (18 ± 6.5mm, p = 0.026 and 16.8 ± 7.3mm, p = 0.0217 respectively). There were no differences in the change of fullness (p = 0.69) or prospective FI (p = 0.17) between HIEX or rest.
Correlations:

Absolute testosterone values were not significantly correlated to the subjective mean appetite score at either rest ($r = 0.06, p = 0.78$) or HIEX ($r = -0.07, p = 0.71$). Testosterone during HIEX was correlated to PYY ($r = 0.54, p = 0.007$) but not at rest ($r = 0.05, p = 0.84$). In addition, absolute testosterone levels during rest and HIEX were not correlated with ghrelin (REST; $r = 0.2, p = 0.32$) and (HIEX; $r = 0.2, p = 0.32$), or GLP-1 (REST; $r = 0.1, p = 0.7$) and (HIEX; $r = 0.01, p = 0.95$). None of the appetite hormones correlated with subjective appetite. Mean subjective appetite during rest and HIEX was not associated with ghrelin (REST; $r = 0.005, p = 0.98$) and (HIEX; $r = 0.03, p = 0.88$), PYY (REST; $r = -0.28, p = 0.19$) and (HIEX; $r = 0.09, p = 0.64$), or GLP-1 (REST; $r = 0.17, p = 0.43$) and (HIEX; $r = -0.25, p = 0.21$) respectively.

Change from baseline in testosterone during rest and HIEX was not correlated with change in GLP-1 (REST; $r = -0.16, p = 0.5$) and (HIEX; $r = -0.02, p = 0.95$), ghrelin (REST; $r = -0.15, p = 0.49$) and (HIEX; $r = -0.14, p = 0.67$) or PYY (REST; $r = 0.07, p = 0.78$) and (HIEX; $r = 0.29, p = 0.41$) respectively. Furthermore, change in testosterone during both rest ($r = -0.04, p = 0.9$) and HIEX ($r = -0.05, p = 0.87$) was not correlated to mean appetite score change.

6.5 Discussion

The results of this preliminary study show that HIEX acutely increased testosterone and reduced appetite and ghrelin in adolescent males. However, the increases in testosterone did not correlate with changes in appetite or appetite hormones.

The results of this study demonstrate for the first time that acute bouts of aerobic activity increase plasma testosterone levels in teenage males. Research examining the effect of exercise in boys has focused primarily on longitudinal interventions/exercise programs, and it has been observed that training in young individuals may modify sex hormone responses [341, 342]. The majority of previous studies reported chronic increases in testosterone. Though prior research on adolescent males has shown an acute testosterone increase with anaerobic training methods such as plyometrics and resistance training [343] or sprinting [345], no increases have been shown with high-intensity aerobic modalities. Prior research by Hackney et al. indicated that there were no significant differences between pre-exercise and post exercise testosterone levels in late-pubertal males who performed 20 minutes of
incremental exercise to exhaustion using a cycle ergometer [346]. This study suffered some major limitations, in particular the lack of a control session. Furthermore, the males were only fasted for three hours before testing which could impact the baseline testosterone levels as we and others have previously shown that food intake influences acute changes in testosterone [10, 380]. Additionally, testosterone was measured using immuno-chemoluminescence which may not be a reliable method to detect small changes in testosterone as compared to the LC-MS or GC-MS [212].

The physiological mechanisms accounting for the increase in testosterone after exercise have not been elucidated. There have been several studies using different forms of exercise which have indicated that the level of LH (gonadotropins) in plasma does not change after exercise [11-13]. However, lactate production may be a factor. With both anaerobic and aerobic exercise, there is an increased production of lactate by glycolysis glycogenolysis within skeletal muscle tissue [347]. In male rats, infusion of lactate resulted in a dose-dependent increase of testosterone synthesis from production by 25-OH-cholesterol in the Leydig cells. In pubertal males, short-term exercise elicits a gradual increase in maximal levels of muscle and plasma lactate but the increases are dependent on pubertal maturation [420-422]. Thus, it has been proposed that lactate directly affects the Leydig cell cAMP production of testosterone and that P450scc is a target of lactate [14]. This hypothesis has been supported by a report showing that exercise-induced lactate production resulted in dose-dependent increases in testosterone and testicular cAMP in rats [348].

The reduction in ghrelin after HIEX in adolescent males when compared to rest is consistent with previous work from our laboratory [401] and that of others in adults [399]. The reductions in ghrelin from HIEX were accompanied with reductions of subjective appetite, though there was no effect on GLP-1 or PYY. Appetite hormones have been hypothesized to be responsible for the post-exercise reduction in appetite in males [267, 423] though direct associations have not been reported. However, this hypothesis was not supported in this study due to a lack of correlations between appetite hormones and subjective appetite.

The decrease in ghrelin suggests it may play a more important role in appetite of male adolescents during HIEX. Others have reported that as a consequence of exercise, ghrelin decreases [424-429] with a concomitant increase in the anorexigenic hormones PYY [428, 430] and GLP-1 [431]. However, as in this study, others have not found the concurrent responses with PYY and GLP-1 [400, 432, 433] suggesting that there is a lack of compelling
evidence any single appetite hormone contributes to appetite suppression during exercise [267].

Furthermore, this is the first study to examine the relationship of appetite with testosterone and exercise in adolescent males. As hypothesized, HIEX increased plasma testosterone, allowing us a further test of previous studies suggesting the decrease in testosterone and appetite after food ingestion indicates it has a role in food intake regulation [380, 403]. However, the lack of relationship between HIEX testosterone and appetite, and the noted increase in testosterone while appetite decreased rather than increased, contrasts with our previous study. Furthermore, though absolute values of testosterone were correlated with PYY during HIEX only, there was no correlation between the changes of these hormones. Taken together, there is little support for testosterone in short-term appetite regulation, leaving unexplained why it responds to food intake. Alternatively, HIEX may not be a suitable model for examining appetite mechanisms.

The lack of an association between testosterone, appetite and appetite hormones may also be explained by disrupted splanchnic blood flow during HIEX. Splanchnic blood flow is increased postprandially to aid digestion, releasing appetite regulating hormones ghrelin, PYY and GLP-1 to signal nutrient availability and satiety [434-438]. In contrast, during HIEX, splanchnic blood flow is decreased from the gut due to the increased oxygen demand of contracting skeletal muscle [439].

Furthermore, more frequent blood measures during the exercise sessions may have provided a greater picture of testosterone change and how it may relate to appetite during exercise. However, due to blood sampling restrictions from the University of Toronto Research Ethics Board in adolescents, this was not possible.

6.6 Conclusions

In conclusion, aerobic HIEX acutely increases plasma testosterone concentrations in males but the increase in testosterone from HIEX was not related to appetite or appetite hormones.
<table>
<thead>
<tr>
<th></th>
<th>Δ Rest</th>
<th>Δ HIEX</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Testosterone</strong> (ng/dl)</td>
<td>-97.76 ± 90.4 (^a)</td>
<td>126.99 ± 87.2 (^b)</td>
</tr>
<tr>
<td>GLP-1 (pM)</td>
<td>-0.18 ± 0.4</td>
<td>-0.15 ± 0.5</td>
</tr>
<tr>
<td>PYY (pg/ml)</td>
<td>-4.61 ± 4.7</td>
<td>4.1 ± 3.0</td>
</tr>
<tr>
<td>Ghrelin (pg/ml)</td>
<td>-12.7 ± 23.8 (^a)</td>
<td>-210.41 ± 46.9 (^b)</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>-0.15 ± 0.1</td>
<td>-0.46 ± 0.1</td>
</tr>
<tr>
<td>Insulin (mg/dl)</td>
<td>1.25 ± 5.2</td>
<td>-6.82 ± 6.5</td>
</tr>
</tbody>
</table>

\(^a\) Values are means ± SEM, \(n = 10\) for testosterone, \(n = 15\) for all other hormones. Statistics were performed on change from baseline. Values from the same categorized column in the same row with different superscript letters are significantly different, \(P < 0.05\).
Table 6.2: Effect of HIEX and rest on plasma testosterone levels between 0 and 65 minutes on each individual subject. Paired sample t-tests were performed to determine differences in change between rest and HIEX.

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>Δ Rest (ng/dl)</th>
<th>Δ HIEX (ng/dl)</th>
<th>DIFFERENCE (ΔHIEX - Δ Rest)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS17</td>
<td>-392.1</td>
<td>134.1</td>
<td>526.3</td>
</tr>
<tr>
<td>AU07</td>
<td>-183</td>
<td>197.1</td>
<td>380.1</td>
</tr>
<tr>
<td>AW02</td>
<td>31.0</td>
<td>124.5</td>
<td>93.5</td>
</tr>
<tr>
<td>BC15</td>
<td>.</td>
<td>.</td>
<td>-</td>
</tr>
<tr>
<td>BT03</td>
<td>.</td>
<td>.</td>
<td>-</td>
</tr>
<tr>
<td>CN05</td>
<td>248.6</td>
<td>935.5</td>
<td>686.6</td>
</tr>
<tr>
<td>JH01</td>
<td>-26.8</td>
<td>220.8</td>
<td>247.6</td>
</tr>
<tr>
<td>JJ10</td>
<td>.</td>
<td>99.5</td>
<td>-</td>
</tr>
<tr>
<td>KP14</td>
<td>47.6</td>
<td>.</td>
<td>-</td>
</tr>
<tr>
<td>KR16</td>
<td>71.3</td>
<td>157.8</td>
<td>86.5</td>
</tr>
<tr>
<td>NC12</td>
<td>-795.6</td>
<td>-369.6</td>
<td>426.0</td>
</tr>
<tr>
<td>NH09</td>
<td>-150.3</td>
<td>-82.4</td>
<td>67.9</td>
</tr>
<tr>
<td>SJ18</td>
<td>417.8</td>
<td>102.9</td>
<td>-314.8</td>
</tr>
<tr>
<td>TG04</td>
<td>.</td>
<td>-76.4</td>
<td>-</td>
</tr>
<tr>
<td>VL06</td>
<td>-281.0</td>
<td>80.1</td>
<td>361.1</td>
</tr>
</tbody>
</table>

| MEAN DIFFERENCE (ΔHIEX - Δ Rest) | 256.1 ± 90.1 ng/dl<sup>1</sup> |

<sup>1</sup>The mean increase of testosterone change was significantly higher (p = 0.019) following HIEX when compared to the mean decrease from rest with a difference of 256 ± 90.1ng/dl between HIEX and rest. All values are means ± SEM, n = 10.
Figure 6.1 - Experimental protocol as described in Hunschede et al. [402]. Subjects arrived in the laboratory after a 12h fast and were randomly assigned to either rest or high intensity exercise (HIEX).
Chapter 7

GENERAL DISCUSSION
7 GENERAL DISCUSSION

The hypothesis that testosterone has an acute response to food intake and HIEX, and has interactive relationship with the short-term regulation of appetite and food intake in adolescent males was partially supported by these studies (Figure 7.1). They are the first to show an acute response of testosterone to food ingestion and HIEX, but did not demonstrate that the testosterone response has an interactive relationship with short-term food intake and appetite regulation. Testosterone was suppressed by both protein and glucose consumption, though the suppression was more sustained after protein (Chapters 4 and 5) and was elevated after high-intensity aerobic cycling when compared with rest (Chapter 6). The responses of plasma testosterone were not associated with a decrease in appetite and FI or appetite-related hormones.

Figure 7.1- Summary of experimental results. Consumption of both protein and glucose resulted in decreased testosterone levels and the decrease persisted after protein intake when compared to glucose (Study 1 and 2). Testosterone was elevated after high-intensity aerobic cycling when compared with rest (Study 3). Plasma testosterone response was not associated with appetite or food intake (FI).
To date, the only study published on single-macronutrient effect of testosterone depression in humans was performed on adult males looking exclusively at glucose with no calorie-free control [10]. The findings of chapter 5 confirm that protein as well as glucose intake diminishes acute testosterone levels but adds the novel finding that this occurs in adolescent males. Additionally, the decrease of testosterone was greater in normal weight than overweight subjects showing that their significantly lower testosterone levels at baseline diminished the effect of food intake. It has been previously established that obese males have lower testosterone at morning than normal weight males [388]. The greater decrease of testosterone caused by protein may be initiated by the intake of amino acids, particularly leucine, which stimulates rapamycin (mTOR) signaling and subsequent protein synthesis independent of insulin [389]. In animal studies, leucine also inhibits adenosine monophosphate-activated protein kinase (AMPK) [391], which is upstream of mTOR and prevents protein biosynthesis [392] in addition to inhibiting androgen receptor (AR) mRNA expression in cancer cells [393]. Leucine induced inhibition of AMPK through protein intake may increase AR expression leading to increased testosterone intake into the muscle tissue from the plasma thereby lowering plasma testosterone levels. The influx of testosterone into the muscle serves to stimulate muscle protein synthesis by regulating nuclear gene expression [103, 104]. Though an attractive explanation, this is only speculative, as this current study cannot determine the direct pathways responsible for the observed changes of testosterone.

This research is also the first to show an acute increase of testosterone after intervals of HIEX aerobic training in this population and is consistent with the current body of literature showing increases of testosterone in teenage males after acute bouts of anaerobic and weight bearing exercise [343, 345]. It is unique in that the exercise applied was aerobic and non-weight bearing which may be a more accessible form of exercise training for overweight adolescent males. Thus, the mode of exercise may be less important than the intensity for the increase in testosterone production in pubertal males; greater lactate production increases Leydig cell cAMP production of testosterone in a dose-dependent manner [14, 348]. Furthermore, the reduction in appetite after HIEX in adolescent males is in agreement with previous findings [401], though these did not correlate to observed changes in testosterone. A direct effect of appetite increasing from chronic elevated testosterone levels has been previously established [331-333, 379] but Chapter 6 is the first study of its kind to show that acute elevated testosterone levels from exercise are not related to post-exercise appetite in adolescent males.
Overall, the results of this research suggests that testosterone does not have an interactive relationship in determining acute food intake, and it remains unclear whether a relationship exists between testosterone, appetite regulation and energy balance in adolescent males. However, there are a number of limitations to consider when interpreting the result of these studies. First, these studies focused on short-term testosterone responses which may have limited application given that testosterone primarily exerts its effects through chronic exposure, thus chronic levels of testosterone are more relevant to its physiological role in modifying. However, there is growing evidence that androgens may exert acute changes on other structures and systems such as altering intracellular calcium regulatory mechanisms [115] and rapid targeting of cardiovascular structures [118-123]. Second, fat intake was not assessed or compared with glucose and protein intake. There is paucity of data on the direct effect of acute fat intake on testosterone levels and this is likely because of palatability issues. One study found no difference in the effect of fat using heavy whipping cream when compared with a liquid dextrose beverage [440]. Furthermore, the type of fat may impact the change seen in testosterone. Meals containing plant-based fat decreased post-prandial testosterone levels more than those with animal fat [375]. A third limitation of this research was the preparation of the experimental beverages; 1g/kg bodyweight of glucose monohydrate or 1g/kg bodyweight of whey protein isolate powder. This meant that there was a caloric discrepancy given that protein powder is not composed of pure protein. Independent third-party analysis of the protein demonstrated 90.4% of the powder was protein, with 5.7% moisture, 2.2% ash, 1.18% fat, and 0.6% carbohydrates. Given these percentages, the protein drink is 3.74 kcal/kg bodyweight and the glucose drink is 4 kcal/kg bodyweight with control being 0 kcal/kg bodyweight. Since the mean of our overweight subjects was 88.5kg, this presents a difference of approximately 23 kcal between the glucose and protein treatments (354 kcal glucose; 331 kcal protein). Previous work comparing a 300kcal preload to a 600kcal preload showed little difference between the two with subsequent subjective appetite and ad libitum energy intake in a buffet meal [441]. Finally, given that most research has examined chronic exposure of exogenous testosterone in animals, the change in testosterone in Chapter 5 and 6 may not have been great enough or sustained long enough to effect appetite and food intake.

A significant strength of this research was provided by the development and use of GC-MS for measurement of testosterone in Chapter 5 and 6. It was a modification (Appendix 2) of a method [181] considered to be the gold standard compared with past research using RIA and
ELISA to detect acute testosterone change. This method allows for detecting modest acute changes of testosterone in adolescent males who have normal pubertal fluctuations of testosterone in addition to expected diurnal rhythms. This may have been a limiting factor in the past for measuring acute change in this population.

This research brings forth novel physiological concepts that could support the currently accepted recommendations of exercise and post-exercise nutrition in adults and may extend them to adolescent males. The International Society of Sports Nutrition (ISSN) recommends ingestion of a post-exercise carbohydrate and protein beverage to maintain a favourable anabolic hormone profile, heighten muscle glycogen recover and mitigate change in muscle damage markers [442]. Combined with findings from animal research, this current research lends strength to the idea that in adolescent males, high intensity exercise increases plasma testosterone (Chapter 6), it is proposed that consumption of a glucose and protein beverage (Chapter 4 & 5) may drive increased plasma testosterone into damaged muscle tissue, possibly through mTOR signaling and increased AR expression after protein intake [389, 393]. Though the exact mechanism could not be determined through the current research presented herein and the fate of postprandial testosterone in adolescent males remains to be determined, this novel concept provides new important questions to pursue.

7.1 Significance and Implications

The rise in adolescent obesity and the attenuation of testosterone production from excess adiposity necessitates a greater understanding of how testosterone is related to energy balance, appetite and food intake. More insight into the association of nutrition and testosterone physiology was provided by these studies.

In addition to garnering a greater understanding of testosterone physiology and its relation to adolescent male growth and nutrition, this research, combined with findings from animal physiology, proposes new concepts for validation of current sports nutrition recommendations for post-exercise protein and glucose ingestion. This research may add to ongoing evidence of the importance of high intensity exercise and post-workout protein consumption for promoting muscle growth. Though the research presented here does not directly address sports nutrition, the observed diminished testosterone levels after a high-
protein beverage and increased testosterone from high-intensity exercise suggest this response requires further research to support current recommendations.

7.2 Conclusion

Acute testosterone concentrations are affected by food intake and exercise in adolescent males, but a relationship of this response to appetite and food intake was not established.
Chapter 8

FUTURE DIRECTIONS
8 FUTURE DIRECTIONS

Future research should consider examining the effect of testosterone dynamics and change over a longer period to determine if testosterone plays a role in appetite regulation of adolescent males. Though previous work has established a role of testosterone in food intake regulation [331-333, 379], the acute nature of this current research may indicate that testosterone mediates appetite and food intake through long-term exposure. Testosterone typically targets cell nuclei to effect transcription leading to protein synthesis, a process that takes several hours with greater changes occurring through chronically elevated levels that extend well beyond the time period of this research [107-112]. Studies in adult males have consistently demonstrated that the scale of increased lean muscle tissue and decreased fat mass from supraphysiological doses of testosterone is correlated with the prevalent testosterone concentrations [134] and that significant increases of lean muscle tissue occur after 20 weeks of regular dosing [137] supporting the chronic effects of testosterone.

Further research should also observe adolescent males with hypogonadotropic hypogonadism who are receiving testosterone injections to stimulate pubertal development as this may be informative. Studying the testosterone treatment response of this population would help determine whether testosterone plays a role in both acute and long-term appetite and food intake in adolescent males and, unlike Chapter 4, 5 and 6, demonstrate a direct effect of testosterone through exogenous administration.

Furthermore, it would be of interest to determine if intake of protein and/or glucose changes the increase of testosterone from high-intensity exercise and the role of lactate. A study combining Chapter 5 and 6 measuring HIEX followed by consumption of protein and glucose may be proposed. Intake of protein immediately after intense exercise is recommended for stimulus of muscle protein synthesis as recommended by the ISSN [442]. Whether this post-exercise postprandial protein synthesis is related to changing levels of testosterone remains unknown. Additionally, as lactate has been shown to play a role in testosterone production [14, 348], demonstrating a direct relationship of HIEX lactate production and testosterone could help guide exercise designs aimed at improving testosterone levels.

Finally, future research should examine sex differences in adolescent populations that could help provide dietary and physical activity guidelines which promote healthy development into adulthood. Given the role estradiol may play in appetite [443] and the changes that occur
from exercise that are not accounted for through weight loss [444], a study of the interaction between food intake and exercise and estradiol in adolescent females may be informative.
Chapter 9

REFERENCES
9 REFERENCES


357. Patel, B.P., et al., *Obesity, sex and pubertal status affect appetite hormone responses to a mixed glucose and whey protein drink in adolescents.* Clin Endocrinol (Oxf), 2014.


Chapter 10

APPENDICES
APPENDIX 1. Pubertal Assessment Flow Chart

Figure A2.1- Proposed pubertal cut off values for hormonal assay values and testicular volume. Luteinizing Hormone values are derived from the work of Chada et al. and Resende et al. [204, 210]. Luteinizing hormone is the most sensitive indicator of onset of puberty, however, if values fall between 0.12 - 0.44 IU/L and 1.6 – 2.39 IU/L, secondary measures of testosterone become necessary to determine the stage of puberty of the subject. Testosterone values are derived from Mouritsen et al. and Konforte et al. [214, 215] using the LC/GC MS methods and IA method respectively. Tertiary measures of testicular volume become necessary if the values derived from the secondary measure of testosterone fall between 35-150 ng/dl. Pre-pubertal values are defined as those < 3.0 ml, whereas values falling between 3.0-12.0ml indicate onset of early puberty and mid-puberty. A testicular volume of >12ml can be defined as late/post puberty.
APPENDIX 2. GCMS Method Development and Validation

For measurements of total serum testosterone most clinical laboratories use ELISA methods. There were different ELISA kits available in market for serum testosterone assay. Like all ELISA procedures, there are some limitations of the testosterone ELISA kits. Recently, several research laboratories developed Liquid Chromatography-Tandem Mass Spectrometry (LC-MS) assay of serum testosterone. This new mass spectroscopy assay techniques and some improved radio immunoassay (RIA) methods for testosterone assay exposed the limitations of the market available ELISA methods for serum testosterone assay. ELISA techniques measuring testosterone have some disadvantages such as low precision, cross-reactivity, limited linear range and poor correlation. Relatively low circulating testosterone concentrations especially in children and women lead to limited precision, unacceptable cross-reactivity and result in positive bias [445].

The most important limitations of all the ELISA assays of serum testosterone become obvious when used in low and high serum testosterone samples. Christina Wang et al [187] findings were supported by several laboratories afterwards and showed can be seen from Table 4 of that article that 19.8, 25.7, 39.6, 39.6, 48.5, and 50.4% of the samples fell outside the 20% range of the LC-MSMS generated serum T value by DPC-RIA, RocheElecsys, Ortho Vitros-Eci, HUMC-RIA, Immulite and Bayer, respectively - all commonly used for clinical measurement of testosterone. This difference was especially noted in the samples with testosterone values less than 100 ng/dl obtained by the six different immunoassays, the majority (55.5–90.0% of the samples) fell outside the 20% range of those obtained by LC-MS. On the other hand, linearity and mean values were calculated from serial dilution of the highest calibrator (16;8;4;2;1;0.5;0.25;0.125;0.06;0.03 ng/mL). Even when the concentration is very low (1 ng/dL) or high (1600 ng/dl) the linearity is not affected.

Wang et al demonstrated that the ELISA methods commonly used in different clinical laboratories gave serum testosterone levels ranging from a low of 200 ng/dl to the highest maximum 420 ng/dl. But the introduction of RIA and LC-MS changed the upper limit of serum testosterone level to even 1500 ng/dl.

The LC-MS assay underwent vigorous validation with a linear calibration curve spanning 20–2000 ng/dl, and had accuracy between 96.6 and 110.4% and precision of less than 10% at all points. The range of serum Testosterone values obtained in 17 normal men ages 18–50 yr in this study was 302–905 ng/dl by the LC-MSMS method [445].

The DPC-RIA (DPC-Coat-a-Count) is the most common RIA used in hospital or reference laboratories and appears to show the best agreement with serum Testosterone values measured in male serum by LC-MSMS. The normal range given by the manufacturer for this assay (286–1510 ng/dl) had a similar low male reference range as other methods but with an extremely high upper limit. This suggests that the adult male range might not have been generated by each laboratory and both the lower and the upper limit of the reference range might have to be adjusted. The Bayer Centaur assay on the other hand showed the reference range for adult men with this instrument is reported as 241–827 ng/dl – the upper limit of this assay was almost half of that determined by RIA or LC-MS methods. This is mostly because of the much better linearity of RIA or LC-MS methods compared to the ELISA methods [446].
A GC-MS modified stepwise procedure was developed by Fitzgerald et al. and utilized for study 2 and 3[181]. The procedure was carried out on an Agilent 5977A single-quadruple mass spectrometer coupled to an Agilent 7890B gas chromatograph (Agilent Technologies, Mississauga, ON) in negative chemical ionization mode at the Department of Nutritional Sciences at the University of Toronto and was validated by Dr. Diptendu Chatterjee, Dr. Richard Bazinet and Scott Lacombe. All extractions and sample preparations were performed by Alexander Schwartz and Dr. Chatterjee and GC-MS analyses were run by Scott Lacombe.

After completing analysis of the samples, we wanted to ensure that we were getting real values and not overestimating testosterone levels in our subjects. We sent out stock testosterone standards of 200, 600 and 1200ng/dl to the rapid response laboratory at The Hospital for Sick Children (HSC) (Table A3.1 and Figure A3.1). This analysis indicates that although there is excellent reliability, there is discordance at higher levels between HSC standards and that of our own.

<table>
<thead>
<tr>
<th>Sample ID (T)</th>
<th>Testosterone (ng/dl)</th>
<th>DNS GCMS values (nmol/l)</th>
<th>HSC LCMS Values (ng/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>7</td>
<td>201.73</td>
</tr>
<tr>
<td>2</td>
<td>600</td>
<td>16</td>
<td>461.10</td>
</tr>
<tr>
<td>3</td>
<td>1200</td>
<td>30</td>
<td>864.55</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>7</td>
<td>201.73</td>
</tr>
<tr>
<td>5</td>
<td>600</td>
<td>16</td>
<td>461.10</td>
</tr>
<tr>
<td>6</td>
<td>1200</td>
<td>30</td>
<td>864.55</td>
</tr>
</tbody>
</table>

Table A3.1- Comparison of stock standards given to The Hospital for Sick Children Rapid Response Laboratory with the Department of Nutritional Sciences GCMS values from the same standards.
Figure A3.1- Comparison of stock standards given to The Hospital for Sick Children Rapid Response Laboratory with the Department of Nutritional Sciences GCMS values from the same standards. Note that the values from HSC do not cross zero due to linearity breaks at higher values.
APPENDIX 3. Study 2 Sub-Analysis of Pre-Early Pubertal Subjects

Results for Pre-Early Subjects from Chapter 5: Acute decrease in serum testosterone and appetite after glucose and protein beverages in adolescent males (Experiment 2).

Participant characteristics are presented in Table A4.1. A total of 11 adolescent males age 9 to 13 that were classified in the pre-early pubertal stages completed the study and were included in this secondary analysis. Four were overweight/obese and were approximately 44% higher in body weight with a five-fold higher fat mass and 25% greater FFM compared to the 7 normal weight participants.

Average baseline levels of appetite- and sex-related hormones are shown in Table A4.2. Overweight/obese participants had higher fasting FSH and lower fasting active ghrelin than the normal weight participants.

Effects of treatments over time on responses in hormone and glucose concentrations

A. Sex hormones
   a) Mean responses

   Mean plasma testosterone concentrations (Table A4.3) were detectable in 5 subjects. Plasma testosterone concentrations were not affected by time (F = 2.33, p = 0.16) or weight status (F = 0.01, p = 0.91) but were affected by treatment (F = 8.22, p = 0.012) with no interactions. The glucose beverage decreased plasma testosterone by an average 34.1% (29.9 ± 9.7 ng/dl, p < 0.0043) compared to control (45.4 ± 9.8 ng/dl). The protein beverage decreased testosterone by 21% in comparison to control, though this was not statistically significant (36.1 ± 9.8 ng/dl, p < 0.11). There was no difference between the effect of protein vs. glucose (p = 0.15) (APPENDIX Figure 1).

   b) AUC responses

Due to the low testosterone values in the pre-early puberty subjects, the AUCs of testosterone could only be determined in one subject for all three of the experimental sessions and were therefore not included in the results.

B. Glucose, Insulin, GLP-1, and Ghrelin

   a) Mean responses

   Plasma glucose concentrations were affected by treatment (F = 19.85, p < 0.0001) and time (F = 7.53, p = 0.0011). Plasma glucose concentrations were higher after the glucose treatment when compared to both protein and control beverages (p< 0.0001) with no differences between protein and control treatments.
Plasma insulin was affected by time ($F = 8.42, p = 0.0005$) and treatment ($F = 54.14, p < 0.0001$). Significant interactions were found in time×treatment ($F = 5.29, p = 0.0009$), treatment×weightstatus ($F = 15.07, p < 0.0001$) and treatment×time×weightstatus ($F = 3.6, p = 0.01$). Plasma insulin concentrations over 65 minutes were highest after glucose consumption when compared to both protein ($p <0.0001$) and the control ($p<0.0001$). Protein also caused a greater increase of insulin when compared to control ($p = 0.0008$). In response to the glucose treatment only, overweight subjects had a greater insulin increase than normal weight ($p = 0.0002$).

GLP-1 was not affected by treatment ($F = 1.92, p = 0.15$), time ($F = 0.61, p = 0.55$) or weight status ($F = 0.21, p = 0.65$). There were no significant interactions between treatment×time ($F = 0.88, p = 0.48$), time×weight status ($F = 0.42, p = 0.66$) or treatment×time×weight status ($F = 0.39, p = 0.82$). GLP-1 (active) average concentrations were similarly increased by glucose ($6.2 ± 0.5pM$), protein ($7.9 ± 0.6pM$) and control beverage ($6.8 ± 0.6pM$) ($p< 0.0001$).

There was an effect of treatment ($F = 21.71 p < 0.0001$) on mean active ghrelin, with no effect of time ($F = 0.56, p = 0.57$) or weight status ($F = 2.03, p = 0.16$). Ghrelin (active) was decreased by glucose ($p < 0.001$) and protein ($p < 0.001$) when compared to control, but there was no difference in ghrelin decrease between the glucose and protein ($p = 0.09$).

b) AUC response

Glucose AUC was greater after the glucose treatment ($p < 0.05$) when compared to protein and control. Additionally, AUC glucose was lower after protein treatment when compared with control ($p = 0.019$).

Insulin AUCs were larger after protein and glucose treatments ($p< 0.01$) when compared with the control. However, the AUC of insulin after glucose was higher than after protein ($p = 0.001$). AUC for insulin was greater in overweight than in normal weight participants after the glucose treatment only ($p = 0.012$).

There was no effect of treatment ($F = 0.8, p = 0.47$) or weight status ($F = 0.01, p = 0.91$) on GLP-1 AUC. Additionally, there were no treatment×weight status interaction ($F = 0.34, p = 0.72$).

Ghrelin AUC was lower ($p< 0.05$) but similar after both the protein and glucose treatments when compared with the control (Table A4.6). There was no treatment×weight status ($F = 0.11, p = 0.05$).

C. Appetite

a). Mean response

Overall, there was a time effect ($F = 4.26, p = 0.018$) but no treatment effect ($F = 0.92, p = 0.4$) on appetite (Table A4.5). Regardless of treatment, appetite was lower ($p = 0.011$) at baseline ($63.9 ± 3.0mm$) when compared with 65 min ($71.3 ± 3.5mm$). In addition, appetite was also lower at 20 min ($65.6 ± 2.8mm$) when compared to 65 min ($p = 0.02$).

c) AUC response.
There was no difference in the average appetite score AUCs due to treatment (p = 0.57) or weight status (p = 0.2).

**D. Food Intake**

There was no effect of the treatment (F = 1.78, p = 0.2) or weight status (F = 0.42, p = 0.5) on total caloric intake. Mean FI for the control, glucose and protein treatments were 839.3 ± 134.4 kcal, 744.2 ± 128.1 kcal and, 706.0 ± 121.5 kcal, respectively. There was no effect of treatment (F = 1.8, p = 0.19) or weight status (F = 0, p = 1.0) on FI corrected for FFM (kcal/kg). Mean intakes for the control, glucose and protein treatments were 29.6 ± 4.8, 26.3 ± 4.4 and, 24.5 ± 4.0/ kg, respectively.

**Correlations:**

Post treatment mean testosterone concentrations over time (Table A4.3) did not correlate with average appetite (r = -0.06, p = 0.7), desire to eat (r = -0.1, p = 0.6), prospective FI (r = 0.06, p = 0.7) or fullness scores (r = 0.12, p = 0.5) (Table 5). No correlation was found with FI at the meal.

AUCs for average appetite were positively correlated with food intake expressed relative to FFM (r = 0.68, p < 0.0001) and total caloric intake (r = 0.67, p < 0.0001). Appetite AUCs did not correlate to GLP-1 AUCs (r = -0.1, p = 0.6) or to AUCs of glucose (r = -0.07, p = 0.7), ghrelin (r = 0.17, p = 0.35), and insulin (r = -0.07, p = 0.7). No within treatment correlations were found.

The AUCs for LH were positively associated with caloric intake expressed relative to FFM (r = -0.5, p = 0.04) but were not associated with subjective appetite (r = -0.42, p = 0.09), insulin (r = 0.12, p = 0.66), active ghrelin (r = 0.08, p = 0.8), glucose (r = -0.07, p = 0.8), or GLP-1 (r = -0.06, p = 0.84).
**Table A4.1. Participant characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Normal Weight</th>
<th>Overweight/Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n = 7 )</td>
<td>( n = 4 )</td>
</tr>
<tr>
<td>Age</td>
<td>10.0 ± 0.3</td>
<td>10.8 ± 0.8</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>138.2 ± 2.7</td>
<td>149.4 ± 8.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>31.0 ± 1.7(^a)</td>
<td>55.2 ± 6.8(^b)</td>
</tr>
<tr>
<td>Fat-Free Mass (KG)</td>
<td>26.5 ± 1.0(^a)</td>
<td>35.2 ± 4.5(^b)</td>
</tr>
<tr>
<td>Fat Mass (KG)</td>
<td>4.4 ± 0.7(^a)</td>
<td>20.0 ± 3.4(^b)</td>
</tr>
<tr>
<td>Body Fat %</td>
<td>13.9 ± 1.7(^a)</td>
<td>36.0 ± 3.2(^b)</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>16.2 ± 0.5(^a)</td>
<td>24.5 ± 1.1(^b)</td>
</tr>
<tr>
<td>BMI %ile (WHO)</td>
<td>33.9 ± 10.1(^a)</td>
<td>97.9 ± 0.7(^b)</td>
</tr>
</tbody>
</table>

1All values are means ± SEM, \( n = 11 \). Values in the same row with different superscript letters are significantly different, \( P < 0.05 \).

**Table A4.2. Baseline levels of appetite- and sex-related hormones**

<table>
<thead>
<tr>
<th></th>
<th>Normal Weight</th>
<th>Overweight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n = 7 )</td>
<td>( n = 4 )</td>
</tr>
<tr>
<td>Testosterone (ng/dl)</td>
<td>26.5 ± 1.0</td>
<td>35.2 ± 4.5</td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>2.6 ± 0.2(^a)</td>
<td>5.1 ± 0.8(^b)</td>
</tr>
<tr>
<td>Ghrelin, active (pg/ml)(^2)</td>
<td>355.5 ± 55.9(^a)</td>
<td>309.5 ± 116.6(^b)</td>
</tr>
<tr>
<td>GLP-1, active (pM)</td>
<td>6.2 ± 0.6</td>
<td>4.7 ± 1.2</td>
</tr>
<tr>
<td>Insulin (uIU/ml)</td>
<td>6.2 ± 0.7</td>
<td>9.2 ± 2.6</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>108.8 ± 2.5</td>
<td>102.3 ± 6.0</td>
</tr>
</tbody>
</table>

1Values were calculated by averaging the baseline levels of all three of the sessions for each hormone. All values are means ± SEM, \( n = 11 \). Values in the same row with different superscript letters are significantly different, \( P < 0.05 \). \(^2\)Ghrelin values are higher than that of Mid-Late Puberty. This is in agreement with previous literature [268, 356].
### Table A4.3. Testosterone and luteinizing hormone response to treatments

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>Glucose</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>20</td>
<td>35</td>
</tr>
<tr>
<td><strong>Testosterone</strong>&lt;sup&gt;2&lt;/sup&gt; (ng/dl)</td>
<td>49.1 ±4.5</td>
<td>76.3 ±28.4</td>
<td>34.7 ±0.5</td>
</tr>
<tr>
<td><strong>LH</strong>&lt;sup&gt;2&lt;/sup&gt; (IU/L)</td>
<td>2.2 ±0.2</td>
<td>2.7 ±0.3</td>
<td>2.3 ±0.4</td>
</tr>
</tbody>
</table>

<sup>1</sup>All values are means ± SEM, n = 4. Values in the same row with different superscript letters are different, P < 0.05 (beverage effect using ANCOVA with proc mixed procedure, Tukey’s post-hoc).

<sup>2</sup>Treatment affected testosterone (F = 8.22, p = 0.012).

### Table A4.4. Ghrelin (active), GLP-1 (active), insulin and glucose in response to treatments<sup>1</sup>

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>Glucose</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>20</td>
<td>35</td>
</tr>
<tr>
<td><strong>Ghrelin</strong>&lt;sup&gt;2&lt;/sup&gt; (active) (pg/ml)</td>
<td>303.7 ±61.4</td>
<td>236.2 ±45.5</td>
<td>268.5 ±57.2</td>
</tr>
<tr>
<td><strong>GLP-1</strong>&lt;sup&gt;3&lt;/sup&gt; (active) (pM)</td>
<td>4.0 ±0.7</td>
<td>5.8 ±1.3</td>
<td>6.0 ±1.2</td>
</tr>
<tr>
<td><strong>Insulin</strong>&lt;sup&gt;4&lt;/sup&gt; (pg/ml)</td>
<td>7.0 ±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5 ±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.2 ±1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Glucose</strong>&lt;sup&gt;5&lt;/sup&gt; (mg/dl)</td>
<td>109.4 ±5.8</td>
<td>116.3 ±3.8</td>
<td>112.6 ±6.4</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are means ± SEM, n = 11. Values in the same row with different superscript letters are significantly different, P < 0.05 (beverage effect using ANCOVA with proc mixed procedure, Tukey’s post-hoc).

<sup>2</sup>Treatment (F = 21.71, p = <0.0001) affected ghrelin with no effect of time (F = 0.56, p = 0.57) or weight status (F = 2.03, p = 0.16).

<sup>3</sup>No effect of treatment (F = 1.92, p = 0.15), time (F = 0.61, p = 0.55) or weight status (F = 0.21, p = 0.65) on GLP-1.

<sup>4</sup>Treatment (F = 54.14, p< 0.0001) and time (F = 8.42, p = 0.0005) affected insulin with no effect of weight status (F = 3.25, p = 0.076) in addition to significant interactions of time×treatment (F = 5.29, p = 0.009), treatment×weight status (F = 15.07, p< 0.0001), time×weight status (F= 3.13, p = 0.0497) and treatment×time×weight status (F = 3.6, p = 0.01).

<sup>5</sup>Treatment (F = 19.85, p<0.0001) and time (F = 7.53, p = 0.0011) affected glucose.

### Table A4.5. Mean VAS for subjective appetite in response to the treatments<sup>1</sup>
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Glucose</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>0  20  35 65</td>
<td>0  20  35 65</td>
<td>0  20  35 65</td>
</tr>
<tr>
<td>Appetite (mm)</td>
<td>61.8 ±6.2  66.8 ±7.0  71.1 ±6.0  74.4 ±6.2</td>
<td>66.2 ±3.5  65.5 ±4.8  63.5 ±6.0  70.7 ±5.7</td>
<td>63.6 ±6.2  65.6 ±6.2  63.8 ±7.6  68.6 ±6.6</td>
</tr>
</tbody>
</table>

1All values are means ± SEM, \( n = 11 \). Values based on subjective measure using a100 mm scale composed of four questions anchored at each end with contrasting terms related to desire to eat, hunger, fullness and prospective food consumption. Mean subjective appetite (appetite) is an aggregate score of these four measures.

2There was a significant effect of time (\( F = 4.26, \ p = 0.018 \)) on appetite.

### Table A4.6. Area under the curve (AUC) for testosterone, LH, ghrelin (active), GLP-1 (active), insulin, glucose and AUC mean appetite score in response to treatments 1,2.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Glucose</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LH</strong> (IU/L)</td>
<td>61.9 ±10.4</td>
<td>56.9 ±12.2</td>
<td>68.0 ±8.5</td>
</tr>
<tr>
<td><strong>Ghrelin, active</strong> (pg/ml)</td>
<td>17078.5 ±3229.55a</td>
<td>15140.1 ±2884.6b</td>
<td>11845.3 ±1796.2b</td>
</tr>
<tr>
<td><strong>GLP-1, active</strong> (pM)</td>
<td>370.6 ±73.3</td>
<td>421.9 ±84.1</td>
<td>472.8 ±89.6</td>
</tr>
<tr>
<td><strong>Insulin</strong> (uIU/ml)</td>
<td>413.1 ±55.8a</td>
<td>2745.8 ±604.4b</td>
<td>1489.5 ±231.0f</td>
</tr>
<tr>
<td><strong>Glucose</strong> (mg/dl)</td>
<td>6774.8 ±133.8a</td>
<td>8426.54 ±548.9b</td>
<td>6078.14 ±213.4c</td>
</tr>
<tr>
<td><strong>Mean Appetite Score</strong> (mm)</td>
<td>4180.0 ±341.6</td>
<td>3975.8 ±275.1</td>
<td>3981.7 ±361.2</td>
</tr>
</tbody>
</table>

1All values are means ± SEM, \( n = 11 \). AUC are calculated as change from baseline over 65 min. Values in the same row with different superscript letters are significantly different, \( P < 0.05 \) (beverage effect using ANCOVA with proc mixed procedure, Tukey’s post-hoc).
Figure A4.1. Effects of treatments on plasma levels of testosterone in pre-early pubertal males (means ± SEM, n = 11). Repeated measures ANCOVA with baseline as a covariate was performed followed by post-hoc analysis. Plasma testosterone was decreased after glucose ($p = 0.0043$) when compared to the non-caloric control drink. Different letters indicate significant difference ($p < 0.05$).
APPENDIX 4. Consent Forms

Chapter 5: Acute decrease in serum testosterone after a mixed glucose and protein beverage in obese peripubertal boys (Study 2)

FitzGerald Building, 150 College Street, 3rd Floor
Toronto, ON M5S 3E2

CANADA

The acute effect of protein or carbohydrate intake on testosterone levels and food intake in children and adolescent boys

Study Information Sheet and Parent’s Consent Form

Investigators: Dr. G. Harvey Anderson, Principle Investigator
Department of Nutritional Sciences, University of Toronto
Phone: (416) 978-1832
Email: harvey.anderson@utoronto.ca

Dr. Jill Hamilton, Associate Professor
Department of Paediatrics, University of Toronto
Phone: (416) 813-5115
Email: jill.hamilton@sickkids.ca

Mr. Alexander Schwartz, Ph.D. Student
Department of Nutritional Science, University of Toronto
Phone: (647) 390-2893
Email: alexander.schwartz@sickkids.ca

Ms. Mukta Wad, Lab Manager
Department of Nutritional Science, University of Toronto
Phone: (416) 978-6894
Email: mukta.wad@utoronto.ca
Purpose of Research:

The purpose of this study is to determine the effects of carbohydrate and protein drinks on testosterone levels and food intake in 9-18 year-old boys. This experiment is being conducted through the Department of Nutritional Sciences at the University of Toronto by Alexander Schwartz (PhD student), Dr. G. Harvey Anderson and Dr. Jill Hamilton (Supervisors). Your son will be required to attend one screening session to explain the study in detail and then attend three experimental sessions (to measure testosterone in addition to blood sugar and insulin) conducted over a 3-week period for a total of 4 visits to The University of Toronto. Experimental sessions will last a maximum of 2 hours.

The purpose of our research is to develop an understanding of factors affecting the control of food intake and testosterone level changes in overweight and obese boys. Knowing the determinants of the regulation of food intake on testosterone levels in boys will allow us to understand how various types of food impact appetite and hormone levels in the body.

Study Visits:

1. Screening visit:

For those parents who express interest in having their sons participate, some information about your son is requested by telephone.

You will be asked to attend a screening session at the university research lab so that the researcher will explain the full details of the study. If you agree to consent for your son to participate, and your son also assents to being in the study, we will do some measurements and questionnaires at the screening visit.

At the screening session we will measure your son’s height, weight, and blood pressure. Your son will have measurements of the waist and arms using a tape measure. We will estimate the amount of muscle and fat tissue in your son’s body, using a technique called the
bioelectrical impedance analysis (BIA). BIA is based on the measurement of electrical resistance in the body to a tiny current (that he cannot feel). Your son will be asked to briefly lie down after removing his shoes, socks and any jewelry. Source electrodes are placed on the backside of his right hand and the top of his right foot and at least 5 cm away from to the receiving electrodes, which are placed on the wrist and on the ankle.

Your son will then be asked to rank his preference for pizza that will be served as the lunch meal at each session. In addition, your son will be familiarized with the scales that we will use throughout the study sessions in which he places a pencil mark to describe various questions about hunger and appetite in addition to his preference for pizza.

Your son’s physical activity and eating habits will be assessed with Physical Activity Questionnaire and your son’s eating behaviours will be assessed with the Dutch Eating Behaviour Questionnaire. He will be asked to fill these out at the screening session visit.

The total time for the screening session visit will be approximately 60 minutes.

2. Study visits 1, 2 and 3

Your son will be asked to come to The University of Toronto, for three individual morning sessions, each lasting about 3-4 hours. These sessions will be held on weekends, over three weeks so that each visit is one week apart. Boys should be brought to the laboratory and returned home by parents only.

On each of the three test days, your son will arrive to the laboratory in the FitzGerald Building at 8:45 am. He should not have anything to eat or drink from 9pm the previous night, except for water, which he can drink until 8 am the day of the visit. After 8 am we would ask that he not have anything to eat or drink until the study begins.

On only the first visit, your son will have a short physical exam including an evaluation of puberty. This involves the nurse doing a quick check of the size of your son’s testicles. If your son does not feel comfortable, he will have a choice to report it himself by answering a questionnaire relating to puberty. The boys will be presented with cartoon pictograms of different stages of pubertal development (e.g., pubic hair, genitalia) and the boys will be asked to pick the picture that best represents their stage of puberty. These pictograms have
been used extensively in youth and adolescents. They will also fill out a brief questionnaire about puberty and changes in their bodies. We are checking this as appetite may change during puberty and may be regulated by testosterone levels. If for any reason the boys are not comfortable with this, they have the option of asking their parents to answer the questionnaire and select the pictograms for them. If you have additional questions about this part of the study, before agreeing to participate, you have the option of discussing this with Dr. Jill Hamilton (416-813-5115 or email:jill.hamilton@sickkids.ca). She is a pediatric endocrinologist at SickKids who specializes in growth and puberty.

Upon arrival, during each of the sessions your son will be given a drink (protein and water, a sugar-sweetened beverage or an equally sweet beverage with no calories). After 60 minutes, he will be offered McCain pizza. He will be told that he may eat as little or as much pizza as he likes. The amount of food eaten by your son will be measured.

Throughout the morning, he will also be requested to complete scales on which he places a pencil mark to describe his desire to eat (“Very weak” to “Very strong”), hunger (“Not hungry at all” to “As hungry as I’ve ever felt”), fullness (“Not full at all” to “Very full”), how much food he could eat (“A large amount” to “Nothing at all”). He will also be asked to complete similar scales on how much he likes the drinks and the pizza. He will complete these scales during the information session, in order to become familiar with the questions.

Also at each study session, blood will be sampled and used to measure blood sugar and appetite-controlling (hunger) hormones. Four blood samples (10 ml or approximately 2 teaspoons) will be taken during each experimental session. The total volume of blood collected at one session will be 40 ml (8 teaspoons) and the total volume of blood collected within three weeks will be 120 ml (24 teaspoons). To obtain blood samples, a nurse will insert a catheter (a needle attached to a plastic tube or IV) into a vein in your son’s arm so that only one poke is needed per session to collect all three blood samples. The catheter will remain in his arm and be used to sample blood in small amounts during the session. After the nurse collects the first sample at baseline (0 minutes), your son will consume one of the drinks within five minutes. After he finishes the drink, we will collect a blood sample at 15, 30 and 60 minutes after baseline. After the third blood measure, your son will be allowed to eat as much pizza as he wishes for 20 minutes.

All youth and adolescents will be fully supervised during the study sessions. They will be engaged in age appropriate entertainment (as distraction) e.g.: reading, puzzles, cards, before lunch. The study session will end shortly after the pizza meal.

Confidentiality:
Records relating to participants will be kept confidential in a locked cabinet in the Department of Nutritional Sciences and no disclosure of personal information of the boys or parents will take place except where required by law. Participants will have a code and a number that will identify them in all documents, records and files to keep their name confidential. All data will be entered into Microsoft Excel files, available only to investigators. Each participant will have a file, also only available for investigators. All blood samples will be stored in a safe and secure refrigerator and may be used for future analyses of other hormones related to appetite regulation. All forms and printouts will be stored in the individual files and clearly labeled. Data stored outside of a secure server will be electronically encrypted to ensure protection of subject identification. All documents will be kept for a minimum of five years following completion of the study and then securely destroyed with the exception of withdrawn data, which will be destroyed immediately upon withdrawal of the participant. Withdrawal of data will be impossible exactly one year after the final completed session.

Risks:

There is very little risk related to this study. The provided test beverages are commercially available and safe for human consumption. In addition, the pizza that boys will be also asked to consume are prepared hygienically in the kitchen at the time of the session. Boys may feel dizzy following the overnight fast, but this is rare. If this happens, they will likely feel fine once they drink the test beverage provided. There is the possibility of a small amount of bruising, pain and the possibility of infection associated with blood collection, but this risk is minimal as all proper sterilization precautions will be met. The pubertal staging questionnaire may also present some psychological and social risks as the cartoon pictures may potentially be embarrassing. In order to reduce this possible risk, participants will given the option to be in a private room with no other individuals present with the exception of the study nurse.

Benefits:

As some of the factors causing obesity remain unclear, the potential benefits from this study will be a better understanding of the regulation of food intake in youth and adolescents and may help us to provide new recommendations for the prevention of obesity in children and teenagers.

Questions and further information:
Participation is completely voluntary and failure to participate will not have any consequences. Also, you and your son have the option to stop participating or skip any step/question at any time.

If you have any questions or would like further information concerning this research project, please do not hesitate to call: Mr. Alexander Schwartz (647) 390-2893, Dr. Jill Hamilton (416) 813-5115 or Dr. Harvey Anderson at (416)-978-1832.

If you have questions or concerns about your rights as a research participant, please contact Dr. Rachel Zand, 416 946 3389, rachel.zand@utoronto.ca.

Dissemination of findings:

A summary of results will be made available to you to pick up after the study is completed. The summary of the data will include data from all of the participants in this study and will be anonymous.

Consent:

I acknowledge that the research procedures described above and of which I have a copy, have been explained to me and that any questions that I have asked have been answered to my satisfaction. I know that I may ask additional questions now or in the future. I am aware that participation in the study will not involve any health risk to my son.

I understand that for purposes of the research project, if my son or I choose to withdraw from the study at any time, we may do so without prejudice.

Upon completion of each study session, my son will receive a $25 gift certificate to the theatre, bookstore or mall after each of the first three sessions and a $125 gift certificate after completing the fourth session and, if in high school, receive volunteer hours. The final summary and results of the study will be available for me to pick up from the Department of Nutritional Sciences, University of Toronto. I am aware that the researchers may publish the study results in scientific journals, keeping confidential my son’s identity.

I hereby consent for my son, ________________________________, to participate in this study.
(Name of parent or guardian)  

(Signature of parent or guardian)

(Name of witness)  

(Signature of witness)

Date: ______________ (dd/mm/yy)
The acute effect of protein or carbohydrate intake on testosterone levels and food intake in children and adolescent boys

Participant’s Assent Form

This study will help to find out how good various drinks are for boy’s health. My weight, height, and body fat will be measured without pain during the screening visit. I will be asked to fill out a questionnaire that is related to my stage of puberty (changes in my body as I grow up). I will also be asked to be examined by a nurse or to look at some cartoon pictures and pick the one that looks most like me. I can ask my parents to answer these questionnaires and pick the picture for me. I will also be asked to drink a drink, complete special scales to show if I am hungry or full and have four blood samples taken by the trained nurse (one before the drink and three after). The nurse will put in a small IV or tube into the vein with the first poke so that there will be no extra needles needed to collect the blood samples. I will also be provided with a pizza lunch 60 minutes after finishing the drink during each study session (that I will eat in The University of Toronto). I will be provided with the pizzas of my choice and be allowed to eat as much pizza as I would like for 20 minutes. All the experimental sessions will be on weekends, school holidays or summer break, so I don’t need to be absent from school.

I know that my participation in the study will not involve any health risk to me.

I will be asked to come for the study three times, but if at any time I decide to stop participating, that will be O.K and I have the choice to not answer any question at any time. I understand that the information related to me will be securely stored and not be given to anyone from outside who is not engaged with this study. I know that as a “thank you” for my participation, I will receive a $25 gift certificate to the theatre, bookstore or mall after each of the first three sessions and a $125 gift certificate after completing the fourth session. I will also receive volunteer hours for high school after completion of each study session.

“I was present when ______________________________ read this form and gave his/her verbal assent.”
_____________________________ Signature

Name of the person who obtained assent: ________________________________
FitzGerald Building, 150 College Street, 3rd Floor
Toronto, ON M5S 3E2
CANADA

The acute effect of protein or carbohydrate intake on testosterone levels and food intake in children and adolescent boys

Study Information Sheet and Participant Consent Form

Investigators: Dr. G. Harvey Anderson, Principle Investigator
Department of Nutritional Sciences, University of Toronto
Phone: (416) 978-1832
Email: harvery.anderson@utoronto.ca

Dr. Jill Hamilton, Associate Professor
Department of Paediatrics, University of Toronto
Phone: (416) 813-5115
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Purpose of Research:
The purpose of this study is to determine the effects of carbohydrate and protein drinks on testosterone levels and food intake in 9-18 year-old boys. This experiment is being conducted through the Department of Nutritional Sciences at the University of Toronto by Alexander Schwartz (PhD student), Dr. G. Harvey Anderson and Dr. Jill Hamilton (Supervisors). You will be required to attend one screening session to explain the study in detail and then attend three experimental sessions (to measure testosterone in addition to blood sugar and insulin) conducted over a 3-week period for a total of 4 visits to The University of Toronto. Experimental sessions will last a maximum of 2 hours.

The purpose of our research is to develop an understanding of factors affecting the control of food intake and testosterone level changes in overweight and obese boys. Knowing the determinants of the regulation of food intake on testosterone levels in boys will allow us to understand how various types of food impact appetite and hormone levels in the body.

Study Visits:

1. Screening visit:

For those who express interest in participating, some information about you is requested by telephone.

You will be asked to attend a screening session at the university research lab so that the researcher will explain the full details of the study. If you agree to consent to participate we will do some measurements and questionnaires at the screening visit.

At the screening session we will measure you height, weight, and blood pressure. You will have measurements of the waist and arms using a tape measure. We will estimate the amount of muscle and fat tissue in your body, using a technique called the bioelectrical impedance analysis (BIA). BIA is based on the measurement of electrical resistance in the body to a tiny current (that he cannot feel). You will be asked to briefly lie down after removing your shoes, socks and any jewelry. Source electrodes are placed on the backside of your right hand and the top of your right foot and at least 5 cm away from to the receiving electrodes, which are placed on the wrist and on the ankle.

You will then be asked to rank your preference for pizza that will be served as the lunch meal at each session. In addition, you will be familiarized with the scales that we will use
throughout the study sessions in which he places a pencil mark to describe various questions about hunger and appetite in addition to his preference for pizza.

Your physical activity and eating habits will be assessed with Physical Activity Questionnaire and your eating behaviours will be assessed with the Dutch Eating Behaviour Questionnaire. You will be asked to fill these out at the screening session visit.

The total time for the screening session visit will be approximately 60-90 minutes.

2. Study visits 1, 2 and 3

You will be asked to come to The University of Toronto, for three individual morning sessions, each lasting about 3-4 hours. These sessions will be held on weekends, over three weeks so that each visit is one week apart. Boys should be brought to the laboratory and returned home by parents only.

On each of the three test days, you will arrive to the laboratory in the FitzGerald Building at 8:45 am. You should not have anything to eat or drink from 9pm the previous night, except for water, which you can drink until 8 am the day of the visit. After 8 am we would ask that you not have anything to eat or drink until the study begins.

On only the first visit, you will have a short physical exam including an evaluation of puberty. This involves the nurse doing a quick check of the size of your testicles. If your do not feel comfortable, you will have a choice to report it yourself by answering a questionnaire relating to puberty. You will be presented with cartoon pictograms of different stages of pubertal development (e.g., pubic hair, genitalia) and you will be asked to pick the picture that best represents your stage of puberty. These pictograms have been used extensively in youth and adolescents. You will also fill out a brief questionnaire about puberty and changes in your body. We are checking this as appetite may change during puberty and may be regulated by testosterone levels. If for any reason you are not comfortable with this, you have the option of asking your parents to answer the questionnaire and select the pictograms for you. If you have additional questions about this part of the study, before agreeing to participate, you have the option of discussing this with Dr. Jill Hamilton (416-813-5115 or email:jill.hamilton@sickkids.ca). She is a pediatric endocrinologist at SickKids who specializes in growth and puberty.
Upon arrival, during each of the sessions you will be given a drink (protein and water, a sugar-sweetened beverage or an equally sweet beverage with no calories). After 60 minutes, you will be offered McCain pizza. You will be told that he may eat as little or as much pizza as he likes. The amount of food eaten by you will be measured.

Throughout the morning, you will also be requested to complete scales on which you place a pencil mark to describe your desire to eat (“Very weak” to “Very strong”), hunger (“Not hungry at all” to “As hungry as I’ve ever felt”), fullness (“Not full at all ” to “Very full”), how much food you could eat (“A large amount” to “Nothing at all”). You will also be asked to complete similar scales on how much you like the drinks and the pizza. You will complete these scales during the information session, in order to become familiar with the questions.

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All youth and adolescents will be fully supervised during the study sessions. You will be engaged in age appropriate entertainment (as distraction) e.g.: reading, puzzles, cards, before lunch. The study session will end shortly after the pizza meal.

Confidentiality:

Records relating to participants will be kept confidential in a locked cabinet in the Department of Nutritional Sciences and no disclosure of personal information of the boys or parents will take place except where required by law. Participants will have a code and a number that will identify them in all documents, records and files to keep their name confidential. All data will be entered into Microsoft Excel files, available only to investigators. Each participant will have a file, also only available for investigators. All blood samples will be stored in a safe and secure refrigerator and may be used for future analyses of other hormones related to appetite regulation. All forms and printouts will be stored in the individual files and clearly labeled. Data stored outside of a secure server will be electronically encrypted to ensure protection of subject identification. All documents will be
kept for a minimum of five years following completion of the study and then securely
destroyed with the exception of withdrawn data, which will be destroyed immediately upon
withdrawal of the participant. Withdrawal of data will be impossible exactly one year after
the final completed session.

Risks:

There is very little risk related to this study. The provided test beverages are commercially
available and safe for human consumption. In addition, the pizza that you will be also asked
to consume are prepared hygienically in the kitchen at the time of the session. You may feel
dizzy following the overnight fast, but this is rare. If this happens, you will likely feel fine
once you drink the test beverage provided. There is the possibility of a small amount of
bruising, pain and the possibility of infection associated with blood collection, but this risk is
minimal as all proper sterilization precautions will be met. The pubertal staging questionnaire
may also present some psychological and social risks as the cartoon pictures may potentially
be embarrassing. In order to reduce this possible risk, participants will given the option to be
in a private room with no other individuals present with the exception of the study nurse.

Benefits:

As some of the factors causing obesity remain unclear, the potential benefits from this study
will be a better understanding of the regulation of food intake in youth and adolescents and
may help us to provide new recommendations for the prevention of obesity in children and
teenagers.

Questions and further information:

Participation is completely voluntary and failure to participate will not have any
consequences. Also, you and your son have the option to stop participating or skip any
step/question at any time.

If you have any questions or would like further information concerning this research project,
please do not hesitate to call: Mr. Alexander Schwartz (647) 390-2893, Dr. Jill Hamilton
(416) 813-5115 or Dr. Harvey Anderson at (416)-978-1832.
If you have questions or concerns about your rights as a research participant, please contact Dr. Rachel Zand, 416 946 3389, rachel.zand@utoronto.ca.

**Dissemination of findings:**

A summary of results will be made available to you to pick up after the study is completed. The summary of the data will include data from all of the participants in this study and will be anonymous.

**Consent:**

I acknowledge that the research procedures described above and of which I have a copy, have been explained to me and that any questions that I have asked have been answered to my satisfaction. I know that I may ask additional questions now or in the future. I am aware that participation in the study will not involve any health risk to me.

I understand that for purposes of the research project, if I choose to withdraw from the study at any time, I may do so without prejudice.

Upon completion of each study session, I will receive a $25 gift certificate to the theatre, bookstore or mall after each of the first three sessions and a $125 gift certificate after completing the fourth session and, if in high school, receive volunteer hours. The final summary and results of the study will be available for me to pick up from the Department of Nutritional Sciences, University of Toronto. I am aware that the researchers may publish the study results in scientific journals, keeping confidential my identity.

I ____________________________ hereby consent to participate in this study.

__________________________________           ___________________________
(Name of parent or participant)                   (Signature of participant)

__________________________________           ___________________________
(Name of witness)                                                    (Signature of witness)

Date: ____________ (dd/mm/yy)
Chapter 6: Acute increase in plasma testosterone after high intensity cycling and appetite in adolescent males (Study 3)

Recruitment Letter for Parents

The acute effects of exercise induced inflammation on appetite and energy intake in lean boys.

Dear Parent,

The University of Toronto is investigating the physiological and environmental determinants of energy intake regulation on the health of children and young adolescents. We are trying to understand the food intake in children. Our ultimate goal is to find ways to address the problems of overeating and obesity that are becoming a concern among Canadians.

We are asking 12-18 year old male participants to take part in a research study. Your child’s participation is straightforward, height, weight, body-fat, physical fitness and questionnaires regarding puberty, eating behaviour and physical activity levels will be assessed during a screening visit. During the other four experimental sessions, your child will be asked to consume water and complete either 30 minutes of resting or 30 (3 x 10 min + 1:30 min rest) minutes of exercise at a moderate intensity, on four separate mornings. The exercise will be conducted on a recumbent bicycle, followed by 60 min of resting (130 min in total). Within each experimental session, your child will be asked to provide 3 teaspoons (30 ml) of blood samples over the course of 2 hours. A registered nurse will take the blood samples. The study will take place on two weekends at the Warren Stevens Building, Faculty of Physical Education and Health Athletic Centre at 55 Harbord Street (on the southeast corner of Harbord and Spadina, Room WS 2030).

There are criteria for participation that you need to be aware of, the child must:

- be 12-18 years of age and
- be healthy and not be taking medications
- have previously taken ibuprofen without any adverse reactions
As a token of consideration your son will receive a gift certificate after each completed session (movie pass, gift cards to the bookstore or gift vouchers to the mall). He will receive a $25 for the screening session, $60 for each of the other four sessions ($265 in total). Should he choose to withdraw from the study before its completion, he will only be compensated for the sessions he already attended, but not for the remaining sessions. In addition, parents will be reimbursed for travel/parking expenses ($12/session).

The University of Toronto Health Sciences ethics review committee has reviewed this study.

If you would like your son to participate, or to get further information beyond that provided in this letter, please contact Mr. Sascha Hunschede principal investigator at (647) 686-2045 or Dr. G. Harvey Anderson, Professor (416) 978-1832 at the University of Toronto (Department of Nutritional Sciences).

Thank you for your support in this important research.

Sincerely,

Dr. Harvey Anderson, Department of Nutritional Sciences, University of Toronto.

Mr. Sascha Hunschede, Department of Nutritional Sciences, University of Toronto.

Dr. Scott Thomas, Faculty of Physical Education and Health, University of Toronto.
**Study Information Sheet and Parent’s Consent Form.**

The acute effects of exercise induced inflammation on appetite and energy intake in lean boys.

Investigators: Dr. G Harvey Anderson

Department of Nutritional Sciences, University of Toronto
Phone: (416) 978-1832
Email: harvery.anderson@utoronto.ca

Mr. Sascha Hunschede
Department of Nutritional Sciences, University of Toronto
Phone: (647) 686-2045
Email: sascha.hunschede@mail.utoronto.ca

Dr. Jill Hamilton, Associate Professor
Department of Pediatrics, University of Toronto
Phone: (416) 813-5115
Email: jill.hamilton@sickkids.ca

Dr. Scott Thomas
Faculty of Physical Education and Health, University of Toronto
Phone: (416) 978-6957
Email: scott.thomas@utoronto.ca

Mr. Alexander Schwartz
Department of Nutritional Sciences, University of Toronto
Phone: (416) 978-6957
Email: a.schwartz@utoronto.ca
Purpose of Research:

The purpose of this study is to determine the effects of exercise induced inflammation on appetite, energy intake and metabolic markers of appetite in normal weight. This experiment is conducted through the Department of Nutritional Sciences, at the University of Toronto by Dr. G. Harvey Anderson (supervisor), Sascha Hunschede (principal investigator) and Dr. Scott Thomas (co-investigator). You will be required to attend four sessions and one screening testing session conducted over a 4-week period, for a total of 4 visits (4 experimental sessions + 1 visit to measure physiological parameters) to the University of Toronto campus. Each visit will last approximately two hours.

Procedure:

Screening

Fitness Testing/Screening:

If your child expresses interest in participating, information about your child will be requested by telephone, by the principal investigator, Sascha Hunschede (647) 686-2045, Sascha.Hunschede@mail.utoronto.ca). If your child is healthy and does not receive any medications, an information session will be arranged.

You and your child will be asked to attend a screening session at the University of Toronto Athletic Center at 55 Harbord Street so that the researcher will explain the full details of the study. If you agree to consent for your son to participate, and your son also assents to being in the study, we will do some measurements and questionnaires at the screening visit.

During the information session, the researcher will explain the full details and risks of the study. Parents and participants who give consent will sign a consent form. You will receive copies of consent forms and of the study information sheet. If your child wishes to participate, your child’s weight, height, and body fat using painless techniques, will be measured.

We will assess your child’s physical fitness at the Cardiorespiratory Fitness Testing Center located in the Goldring Center of the University of Toronto campus on a seated bicycle using a simple, non-invasive technique. During the test, a facemask will be worn to facilitate the collection of ventilator gases. In addition, your child’s heart rate (Polar Monitor) and mechanical workload will be monitored throughout the test. The test will be conducted on a bicycle and every two minutes the resistance will be increased. The test will be stopped when your child request it (signal is thumbs down) or when you reach your maximum aerobic capacity, or if the supervisor halts the test. The exercise time on the bicycle will be approximately 10 to 15 minutes. Your child will be asked to abstain from eating, drinking caffeine-containing beverages for 10 hours prior to the tests.
There will be four individuals present during the fitness testing process including: a technician who will operate the exercise equipment and computer software, Mr. Sascha Hunschede, principal investigator, Dr. Scott Thomas who is an expert in the area of cardiorespiratory fitness testing in children and a research assistant.

Activity Assessment:

If your child consents to participate in this experiment, he will be asked to fill out a Physical Activity Questionnaire (PAQ) to assess your participation in physical activity. The PAQ will be filled out during each visit to the University of Toronto. The answers will be strictly confidential and will only serve to assist in the analysis of the data collected. Subsequent to the start of the experiment, any relevant changes in health status should be reported as soon as possible to Mr. Sascha Hunschede (principal investigator), Dr. Thomas, or Dr. Anderson.
**Session Visits:**

You and your son will be required to come to the University of Toronto Athletic Centre at 55 Harbord Street after a 10-hour overnight fast for four study visits. Each visit will be separated by one week apart. Participants will receive 2 exercise treatments and 2 resting treatments with either water or 300mg of Ibuprofen, one week apart. Drink treatments will be blinded with 2g cool aid to mask the taste of them Children’s PERRIGO® Suspension. Your child is encouraged to bring homework or reading materials during the 4 experimental sessions. The exercise protocol is described in Exercise Protocol. Please make sure to bring exercise attire for each session.

The treatment order will be randomized:

- 250ml Water with 2g of cool aid and rest (control)
- 250ml Water with 2g of cool aid and 30 min of exercise on a seated bicycle at 70% VO2max.
- 235ml Water with 15ml of Children’s PERRIGO® Suspension (Medical Ingredient: 300mg Ibuprofen)
- 235ml Water with 15ml of Children’s PERRIGO® Suspension (Medical Ingredient: 300mg Ibuprofen) + 30 min of exercise on a seated bicycle at 70% VO2max

**Exercise Protocol:**

Your child will cycle on a seated bicycle, at a high (70% VO2max) intensity, for 30 minutes in 3 x 10 minute periods with 1:30 minute breaks in between.

**Preload treatments:**

Two of the preload treatments will include 300mg Ibuprofen. Ibuprofen is a member of the class of agents commonly known as nonsteroidal anti-inflammatory drugs (NSAIDs). Ibuprofen is commonly used for the temporary relief of minor aches and pains in muscles, bones and joints, headache, fever, the aches and fever due to the common cold or flu, immunizations, toothache (dental pain), sore throat, earache. Ibuprofen was chosen because it has the most favourable gastrointestinal safety profile of all NSAIDs with a very low risk of causing serious events, according to the Food and Drug Administration (FDA) and the Non-prescription Drug Advisory Committee. It also often used by athletes for decrease muscle soreness and better recovery. Exercise itself causes a natural occurring inflammatory response that may affect appetite. Ibuprofen will be given to your child to suppress this inflammatory response. Your child will only be eligible to participate in this study, if he has taken Ibuprofen previously and displayed no adverse reactions to the drug. The dosing recommendation for the age group, in this study, is 400 mg every 4 to 6 hours as needed. In this study we will give one
dose of 300 mg of a child-approved version of ibuprofen Children’s PERRIGO® Suspension to minimize side effects.

*Risks of the preload ibuprofen treatment:*

Common (3-9%) side effects of ibuprofen can include nausea, dyspepsia, gastrointestinal ulceration/bleeding, raised liver enzymes, diarrhea, constipation, nosebleed, headache, dizziness, rash, salt and fluid retention, and hypertension. Ibuprofen may (<1%) cause an allergic reaction as indicated by (wheezing; facial swelling or hives; shortness of breath; shock; fast, irregular heartbeat) if any of these reactions occur, stop the use and get medical help immediately. If any of these reactions occur, the study will be stopped and get medical help will be sought immediately. A full list of side effects is attached with this document.
**Risks of exercise:**

Dr. Scott Thomas has many years of experience assessing physical fitness. The fitness testing process will utilize state of the art equipment. There are minimal risks associated with any workout or maximum exercise test, however these risks can be lowered substantially with proper precautions. Your child’s vital signs will be closely monitored throughout the test and it is important that your child communicates about any symptoms to staff, including chest pain, difficulty breathing, dizziness, nausea, headaches, double vision and neck rigidity.

**Appetite Assessment:**

You will also be requested to complete scales on which they will place a pencil mark to describe their desire to eat (“Very weak” to “Very strong”), hunger (“Not hungry at all” to “As hungry as I’ve ever felt”), fullness (“Not full at all” to “Very full”), how much food you could eat (“A large amount” to “Nothing at all”), sweetness of the drinks (“Not sweet at all” to “Extremely sweet”). They will complete these questionnaires during the information session, in order to become familiar with the test instruments.

**Blood measurements:**

There is minimal risk to your son. Four 10 ml intravenous blood samples will be taken throughout each experimental session, one sample is the equivalent of approximately two teaspoons of blood. A trained nurse will be on sight to conduct the blood sampling and to ensure the safety of our participants. The volume of blood taken presents a minimal risk to subjects. There is a moderate risk of infection from insertion of the catheter or venous puncture. However, a sterile indwelling catheter will be used and the area will be swabbed with alcohol to decrease the risk. There is the possibility of a small bruising, pain and the possibility of infection associated with blood collection. The nurse will offer topical anaesthetic spray to your son, to numb the skin prior to inserting the needle into your son’s vein. There is a small risk of discomfort during the study period. However, your child will be able to stop the study at any point of time.

**Body Composition Assessment:**

**Bioelectrical Impedance Analysis:**

Bioelectrical impedance analysis (BIA), a recently developed technique for measuring body fat content in both adults and children, is simple and painless and is an effective method for measuring body fat. BIA is based on measurement of electrical resistance in the body to a tiny current (that the subject cannot feel). The principle of BIA lies in that muscle mass in the body is a better conductor of electricity than fat which has lesser amounts of water and electrolytes.
Confidentiality:

Confidentiality will be respected and no information that shows your identity will be released or published without your permission unless required by law. Your name, personal information and signed consent form will be kept in a locked filing cabinet in the investigator’s office. Your results will not be kept in the same place as your name. Your results will be recorded on data sheets and in computer records that have an ID number for identification, but will not include your name. Your results, identified only by an ID number, will be made available to the study sponsor if requested. Only study investigators will have access to your individual results. The information you share with me will be kept private except for information that leads me to believe that a Person under the age of 16 has suffered or is at risk physical, sexual harm or neglect. I have a legal duty to report abuse to the Children’s aid society under Ontario’s child and family services act.
Benefits:

Community benefits from this project by potential new strategies that can improve energy homeostasis in children and adults by investigating the effects of fat oxidation during exercise on post exercise food intake. As the causes of obesity remains undefined, the potential benefits from this study will be a better understanding of the regulation food intake in children and might contribute to the prevention of obesity in children.

Questions and further information:

If you have any questions or would like further information concerning this research project, please do not hesitate to call: Sascha Hunschede principal investigator at (647) 686-2045 or Dr. Harvey Anderson, supervisor at (416)-978-1832.

If you have questions or concerns about your rights as a research participant, please contact the Office of Research Ethics, ethics.review@utoronto.ca or at 416-946-5806.

Dissemination of findings:

A summary of results will be made available to you after the study is concluded.

Consent:

I acknowledge that the research procedures described above and of which I have a copy, have been explained to me and that any questions that I have asked have been answered to my satisfaction. I have read and understood the recruitment letter, and information sheet including the drug information sheet related to the risk of taking ibuprofen. I hereby acknowledge that my child has taken ibuprofen previously and did not have any adverse reactions to Ibuprofen or any other painkillers. I know that I may ask additional questions now or in the future. All the risks associated with this study have been explained to me and I am fully aware of the health risks involved in this study.

I understand that for purposes of the research project, if I choose to withdraw consent and/or my son from the study at any time, we may do so without prejudice.

As a token of consideration for participating in research, at each session your son will receive a gift certificate (movie pass, gift cards to the bookstore or gift vouchers to the mall). A $25 gift certificate will be given to your son for the screening session and a $60 gift certificate will be given for each of
experimental sessions. Should your son choose to withdraw from the study before its completion, he will only be compensated for the sessions he attended, but not for the remaining sessions.

The final summary and results of the study will be available for me to pick up from the Department of Nutritional Sciences, University of Toronto. I am aware that the researchers may publish the study results in scientific journals, keeping my identity confidential.

I hereby consent, __________________________________________, to participate in this study.

__________________________________________________________
(Name of guardian or parent of participant) (Signature of guardian or parent of participant)

__________________________________________________________
(Name of witness) (Signature of witness)

Date: ____________ (dd/mm/yy)
APPENDIX 5. Screening Questionnaires

Telephone Screening Questionnaire

Recruitment Screening & Food Acceptability Questionnaire

Eating Habit Questionnaire

Physical Activity Questionnaire

Puberty Questionnaire
5.1. Telephone Screening Questionnaire

Name: ________________________________

Age: ___________ years DOB (d/m/y) ___________ Term baby? Yes / No

Height: ___________ cm Weight: ___________ kg Normal birth weight? Yes / No

Has your child gained or lost weight recently? Yes / No (circle correct answer)

Does your child usually have breakfast? Yes / No

Does your child like (foods that will be used in study)

- Milk/protein smoothies Yes / No
- Sugary Drinks Yes / No
- Pizza Yes / No

Is your child following a special diet? Yes / No

Does your child have food allergies or sensitivities? Yes / No

Health problems? Yes / No

If yes, which problem? ________________________________

Medication/s? Yes / No

If yes, which medication/s? ________________________________

Education: Grade: ___________
<table>
<thead>
<tr>
<th>Question</th>
<th>Yes / No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skipped or repeated grade?</td>
<td></td>
</tr>
<tr>
<td>Learning difficulties/problems?</td>
<td></td>
</tr>
<tr>
<td>Behavioral or emotional problems</td>
<td></td>
</tr>
<tr>
<td>If yes, which problem?</td>
<td></td>
</tr>
<tr>
<td>Include in study?</td>
<td></td>
</tr>
<tr>
<td>If not, why?</td>
<td></td>
</tr>
<tr>
<td>Appointment date:</td>
<td></td>
</tr>
<tr>
<td>Investigator:</td>
<td></td>
</tr>
<tr>
<td>Date:</td>
<td></td>
</tr>
</tbody>
</table>
5.2. Recruitment Screening Information Questionnaire

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>TEST0______</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject Name:</td>
<td>____________________________________________</td>
</tr>
<tr>
<td>Child’s Date of Birth</td>
<td>____________________________________________</td>
</tr>
<tr>
<td>Child’s Date of Birth</td>
<td>____________________________________________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parent 1</th>
<th></th>
<th>Parent 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight:</td>
<td>Kg / lb</td>
<td>Weight:</td>
<td>Kg / lb</td>
</tr>
<tr>
<td>Height:</td>
<td></td>
<td>Height:</td>
<td></td>
</tr>
</tbody>
</table>

__________________________

**Contact Information**

| Address:  | ____________________________________________ |
|-----------|__________________________________________|
| Home Phone #: | ____________________________________________ |

Parent 1 Name: ____________________________________________
Cell Phone #: ____________________________________________
Work Phone #: ____________________________________________
Email ____________________________________________

Preferred Method of Contact
Home Phone ☐  Cell Phone ☐  Email ☐

Parent 2 Name: ____________________________________________
Cell Phone #: ____________________________________________
Work Phone #: ____________________________________________
Email ____________________________________________

Preferred Method of Contact
Home Phone ☐  Cell Phone ☐  Email ☐
Food Acceptability List
Department of Nutritional Sciences, University of Toronto
Pre-meal Beverage, Testosterone and Food Intake in Overweight and Obese boys

Name: ___________________________ Birth Date: ___________________________

BEVERAGE
Please indicate whether you will be able to drink the beverages below:
- Flavoured Protein Smoothie (500 mL) Yes / No
- Flavoured Juice (500 mL) Yes / No
- Flavoured Sweetened Juice (500 mL) Yes / No
  (Circle one Yes OR No)

LUNCH
You will be given a pizza lunch on the day of the study.
For us to provide you with a lunch you will enjoy, please circle what you would like to eat
(circle a, b OR c):
(a) All PEPPERONI pizza (cheese & pepperoni
(b) All CHEESE pizza (3-cheese: mozzarella, cheddar and parmesean)
(c) A COMBINATION of pepperoni & 3-cheese pizza

If you answered (c), please circle what you would like more:
pepperoni OR 3-cheese
(Circle One)

BLOOD
We will require two blood samples for each session in this investigation. Please indicate whether
you will be able to provide us with blood samples.
Yes ☐ No ☐

TANNER STAGING
The study nurse will discuss pubertal Tanner staging with you. Please indicate whether you agree
to have this examination performed.
Department of Nutritional Sciences, University of Toronto
Pre-meal Snacks, Satiety and Food Intake in Children

Body Measurements

Subject Code: ____________________
Date of Birth: ____________________ (dd/mm/yy)
Age: ____________________
Weight: ____________________ Kg  Height ____________________ cm

BIA

| Resistance |  |
| Reactance  |  |
| Body Fat % |  |
5.3. Eating Habit Questionnaire

Dutch Eating Habits Questionnaire
*Please complete sections 1 and 2 and then turn over.*

1. Subject and test details

Name: _____________________________________________________________

Date of birth: ______________________________________________________

Age: _____________________________________________________________

Gender: □ male □ female

Today’s date: ______________________________________________________

2. Your weight, height, etc.

A. Current weight (kg): ______________________

B. Current height (cm): ______________________

C. Has your body weight been constant over the past six months?
   □ yes, my weight did not change much
   □ no, I lost ________ kg
   □ no, I gained ________ kg
   □ no, sometimes I gained weight and sometimes I lost weight

D. Have you ever had an episode of eating an amount of food that others would regard as unusually large?
   □ yes
   □ no

**Please do not mark below this line**

BMI (*please take the age of the child into account*): ______________________

<table>
<thead>
<tr>
<th>DEBQ scale</th>
<th>Raw score</th>
<th>Number of items</th>
<th>Scale score</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emotional eating</td>
<td></td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>External eating</td>
<td></td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Restrained eating</td>
<td></td>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Please turn over >>>>>>*
**Instructions**
Below you’ll find 20 questions about eating.
Please read each question carefully and tick the answer that suits you best. Only one answer is allowed. Don’t skip any answer.
There are no incorrect answers; it’s your opinion that counts.

<table>
<thead>
<tr>
<th></th>
<th>Question</th>
<th>No</th>
<th>Sometimes</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Do you feel like eating whenever you see or smell good food?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>If you feel depressed do you get a desire for food?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>If you feel lonely do you get a desire for food?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Do you keep an eye on exactly what you eat?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Does walking past a candy store make you feel like eating?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Do you intentionally eat food that helps you lose weight?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Does watching others eat make you feel like eating too?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>If you have eaten too much do you eat less than usual the next day?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Does worrying make you feel like eating?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Do you find it difficult to stay away from delicious food?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Do you intentionally eat less to avoid gaining weight?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>If things go wrong do you get a desire for food?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Do you feel like eating when you walk past a restaurant or fast food restaurant?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Have you ever tried not to eat in between meals to lose weight?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Do you have a desire to eat when you feel restless?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Have you ever tried to avoid eating after your evening meal to lose weight?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Do you have a desire for food when you are afraid?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Do you ever think that food will be fattening or slimming when you eat?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>If you feel sorry do you feel like eating?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>If somebody prepares food do you get an appetite?</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**PLEASE CHECK, TO BE SURE THAT YOU TICKED EVERY QUESTION.**
PAST YEAR PHYSICAL ACTIVITY

Check all the activities that you did at least ten times in the PAST YEAR. Include times spent in school physical education classes. Make sure you include all sport teams that you participated in during the last year.

- Aerobics
- Band/Drill Team
- Baseball
- Basketball
- Bicycling
- Bowling
- Cheerleading
- Dance Class
- Football
- Garden/Yard Work
- Gymnastics
- Hiking
- Ice Skating
- Roller Skating
- Running for Exercise
- Skateboarding
- Snow Skiing
- Soccer
- Softball
- Street Hockey
- Swimming (Laps)
- Tennis
- Volleyball
- Water Skiing
- Weight Training
- Wrestling (Competitive)
- Others

List each activity that you checked above in the “Activity” box below, check the months you did each activity and then estimate the amount of time spent in each activity.
5.5. Puberty Questionnaire

Would you say that your growth spurt (height)?
1. there has been no development
2. development has barely begun
3. development is definitely underway
4. development is already completed

And regarding hair growth (under your arms, your pubic hair), would you say that:
1. there has been no development
2. development has barely begun
3. development is definitely underway
4. development is already completed

Have you noticed changes in your skin (e.g. acne)?
1. there have been no changes
2. changes have barely begun
3. changes are definitely underway
4. changes are already complete

Have you noticed that your voice has changed (lowered)?
1. there have been no changes
2. changes have barely begun
3. changes are definitely underway
4. changes are already complete

Have you started to see hair on your face?
1. there have been no changes
2. changes have barely begun
3. changes are definitely underway
4. changes are already complete

Tanner stage exam to be performed by MD or health care practitioner trained in Tanner staging. If subject refuses exam, self-stage with cartoons and tanner beads.
1. Put an 'X' in the box that is closest to the genitalia Tanner stage.

1] The size and shape of the testes, scrotum (the sac holding the testes) and penis are about the same as when you were younger.

2] The penis is a little bit bigger. The scrotum has dropped and the skin of the scrotum has changed. The testes are bigger.

3] The penis has grown longer, the testes have grown and dropped lower.

4] The penis is longer and wider. The head of the penis is bigger, the scrotum is a darker color and bigger. The testes are bigger.

5] The penis, scrotum and testes are the size and shape of a man's.
1. Put an 'X' in the box that is closest to the genitalia Tanner stage.

1 [ ] The size and shape of the testes, scrotum (the sac holding the testes) and penis are about the same as when you were younger.

2 [ ] The penis is a little bit bigger. The scrotum has dropped and the skin of the scrotum has changed. The testes are bigger.

3 [ ] The penis has grown longer, the testes have grown and dropped lower.

4 [ ] The penis is longer and wider. The head of the penis is bigger, the scrotum is a darker color and bigger. The testes are bigger.

5 [ ] The penis, scrotum and testes are the size and shape of a man's.
3. Testicular volume assessment using orchidometer. If the volume is in between two beads, select the smaller volume.

   a. Right testicle:
      CHECK ONLY ONE BOX

         □ 1-2 ml
         □ 3 ml
         □ 4 ml
         □ 5 ml
         □ 6 ml
         □ 8 ml
         □ 10 ml
         □ 12 ml
         □ 15 ml
         □ 20 ml
         □ 25 ml
         □ Undescended
         □ Known to be absent

   b. Left testicle:
      CHECK ONLY ONE BOX

         □ 1-2 ml
         □ 3 ml
         □ 4 ml
         □ 5 ml
         □ 6 ml
         □ 8 ml
         □ 10 ml
         □ 12 ml
         □ 15 ml
         □ 20 ml
         □ 25 ml
         □ Undescended
         □ Known to be absent

   NOTE: If one of the testicles cannot be palpated, the mother should be instructed to contact the child’s health care provider.

Trained medical person doing pubertal assessment: ____________________

Pubertal Assessment Tool Kit – Boys – Trained Medical Assessment 19 June 2013
APPENDIX 6. Study Day Questionnaires

Baseline/Recent Food Intake Questionnaire

Motivation to Eat VAS

Physical Comfort VAS

Treatment and Test Palatability

Test Meal Record
6.1. Baseline/Recent Food Intake Questionnaire

Subject ID: ______________________  Session: ____________

Date: ______________________

Baseline Questionnaire (to be asked by investigator)

1. Have you had anything to eat or drink for 10 - 12 hours?  YES / NO

2. How are you feeling today?

3. Are you taking any medication?  YES / NO
   If yes, please describe briefly ________________________________

Comments/Notes:

Investigator: ______________________
6.2. Motivation to Eat VAS

Visual Analogue Scale
Motivation to Eat

These questions relate to your “motivation to eat” at this time. Please rate yourself by placing a small “x” across the horizontal line at the point which best reflects your present feelings.

1. How strong is your desire to eat?

| Very WEAK | Very STRONG |

2. How hungry do you feel?

| NOT Hungry at all | As hungry as I have ever felt |

3. How full do you feel?

| NOT Full at all | VERY Full |

4. How much food do you think you could eat?

| NOTHING at all | A LARGE amount |

5. How thirsty do you feel?

| NOT Thirsty at all | As thirsty as I have ever felt |
6.3. Physical Comfort VAS

Visual Analogue Scale
Physical Comfort

This question relates to your “physical comfort” at this time. Please rate yourself by placing a small “x” across the horizontal line at the point which best reflects your present feelings.

1. How well do you feel?

<table>
<thead>
<tr>
<th>NOT well</th>
<th>____________________________</th>
<th>VERY Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>at all</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.4. Treatment and Test Palatability

Visual Analogue Scale
Pleasantness

This question relates to the palatability of the drink you just consumed. Please rate the pleasantness of the drink by placing a small “x” across the horizontal line at the point which best reflects your present feelings.

1. How pleasant have you found the drink?

| NOT at all pleasant | VERY pleasant |

This question relates to the palatability of the drink you just consumed. Please rate the sweetness of the drink by placing a small “x” across the horizontal line at the point which best reflects your present feelings.

1. How sweet have you found the drink?

| NOT sweet at all | VERY sweet |

|
### Test Meal Record

- **Subject ID:** ____________  
  **Pizza Preference:** ____________________________

- **Amount of whey for treatment:** ____________ (g)
- **Amount of glucose for treatment:** ____________ (g)

**Amount of sucralose needed for treatment:**

<table>
<thead>
<tr>
<th>Session</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tbody>
</table>

**Investigator:** ____________  
**Date:** ____________

<table>
<thead>
<tr>
<th>Tray 1</th>
<th>Before</th>
<th>After</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepperoni (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-cheese (g)</td>
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<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Tray 2</th>
<th>Before</th>
<th>After</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepperoni (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-cheese (g)</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
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<tr>
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<th>Before</th>
<th>After</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepperoni (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-cheese (g)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>