THE EFFECT OF WHEY PROTEIN ISOLATE ON PLASMA AMINO ACIDS, NITROGEN BALANCE, GLUTATHIONE AND PERFORMANCE DURING ENERGY RESTRICTION IN ATHLETES

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ABSTRACT

This study compared the effects of whey and casein on plasma AA, nitrogen balance (NBAL), glutathione and performance in dieting athletes. Twenty cyclists consumed 40 $g \cdot d^{-1}$ whey (WHEY) or casein (CAS) for 3 wk. On d 18 – 21 subjects restricted intake to 20 $kcal \cdot kg^{-1} \cdot d^{-1}$ plus protein supplement. Apparent NBAL was estimated on d 18 – 21 while postabsorptive and 2 h postprandial plasma AA were measured on d 14 and 21. On d 1, 15 and 22 subjects performed an exercise performance test and provided blood for glutathione analysis. Both groups experienced similar negative NBAL (CAS = -19.7 ± 1.4 g, WHEY = -21.4 ± 2.7 g) during energy restriction. There were trends towards a reduction in performance during energy restriction ($p = 0.073$) and an interaction of group with day ($p = 0.072$). There were significant main effects of state (postabsorptive = 34.5 ± 2.4 μM, postprandial = 37.1 ± 3.0 μM; $p = 0.038$) and day (d 14 = 33.8 ± 2.2 μM, d 21 = 37.8 ± 3.2 μM; $p = 0.008$) on plasma cysteine. There was a significant interaction of state and day on glutamine ($p = 0.002$), as levels increased 1.3% from postabsorptive to postprandial measurements on d 14, but decreased 4.2% on d 21. The absolute change in postabsorptive cysteine from d 14 to d 21 was correlated with NBAL ($r = 0.766$, $p = 0.01$) in CAS but not in WHEY. Plasma glutamine did not correlate with NBAL in either group.

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CHAPTER 1: INTRODUCTION

Introduction

The nutritional supplement industry is a multi-billion dollar per year enterprise, releasing products touted to produce myriad benefits to those who consume them. Many of the purported effects of nutritional supplements remain to be properly tested. Among these are numerous claims to improve athletic performance and/or body composition. Whey, a component of milk leftover as waste in the production of cheese, is an abundant source of protein and is often marketed for consumption by athletes. While much research has been done on the possible ergogenic effects of different varieties of whey (e.g. 20, 24, 51), not all preparations are necessarily equal in their bioactivities.

Immunocal® is a commercially available whey protein isolate produced without the high heat and/or low pH of typical pasteurization methods. According to company literature, the avoidance of these harsh conditions allows the glutamylcysteine dipeptides present in the serum albumin and lactoferrin components of whey, and cystine residues in the former two components and α-lactalbumin, to remain intact in Immunocal®. The availability of cysteine in the body is thought to both limit the synthesis of the antioxidant glutathione (GSH) and possibly have a regulatory effect on body protein metabolism.

Athletes in various aerobic sports may undergo energy restriction to lose weight in an attempt to reduce energy costs and, in turn, improve performance (35). However, during the energy restriction period, individuals may experience reduced physical performance (108, 112) and loss of lean body mass (100, 102, 103, 110, 111). Some individuals look to nutritional methods to counteract these effects.

Some studies suggest that specific plasma amino acid patterns predict body protein loss during catabolic states (32, 44, 118). For example, one research group has repeatedly observed a correlation between low plasma cysteine and loss of lean tissue (31, 62). Consumption of whey protein has been shown to increase plasma cysteine concentrations in certain populations (41).

Another potential benefit of whey protein consumption is increased availability of cysteine for synthesis of the antioxidant glutathione (GSH). Decreased availability of GSH hastened fatigue in rats (65, 98) and limited research in humans suggests that a whey protein supplement is ergogenic for high intensity exercise (66).

Dietary supplementation with Immunocal® may be of particular interest to dieting athletes who would like to maintain performance and reduce loss of lean body mass.

Statement of the Problem

Athletes often restrict energy intake in an attempt to reduce body weight and/or body fat (35). However, individuals undergoing energy restriction may also experience reduced physical performance (e.g. 112) and loss of lean body mass (e.g. 102), consequences in opposition to the goal of energy restriction. Much research has been done on nutritional supplements with which to avoid both of these negative consequences of short-term energy restriction on athletes, without a consensus on one superior product.

Results from several studies have demonstrated a correlation between low plasma cysteine and loss of lean tissue in catabolic states (31). Cysteine, an amino acid present in large amounts in the whey component of milk, is thus thought to play a vital role in the regulation of body protein metabolism. Consumption of a whey protein supplement has been shown in one study to be associated with increased plasma cysteine concentrations in low-birthweight infants (41). Although this population is often at risk for lean tissue loss, it is not necessarily comparable to a healthy population. No studies have been done to investigate the effect of a whey protein isolate supplement, in particular Immunocal®, on plasma cysteine and other amino acid levels and nitrogen balance in healthy athletes undergoing energy restriction. Thus, the effect of whey protein isolate on nitrogen balance in this population remains unknown and warrants further study.

Another potential benefit of whey protein consumption is increased availability of cysteine for glutathione (GSH) synthesis. Decreased availability of this antioxidant has repeatedly been shown to hasten fatigue in rats (e.g. 65). A single study in humans supplemented with whey protein suggested that the supplement increased GSH concentration and was ergogenic (66). This human research was limited, however, by confounding variables and an imperfect experimental design. The results of this study must be verified and possible performance enhancement validated with a test more appropriate to the proposed mechanism of the supplement.

The limited available research suggests that supplementation with whey protein isolate may be of particular interest to dieting athletes who would like to maintain

performance and reduce loss of lean body mass due to the high levels of cysteine in the supplement. The available studies are far from conclusive, however, and further research must be done to validate the ergogenicity of whey protein isolate and to investigate the mechanism for its possible performance enhancing effects.

Objectives

- To determine if Immunocal® supplementation has a different effect than casein supplementation on:
    a. fasted and postprandial plasma amino acid concentrations during energy balance and during weight loss.
    b. loss of body nitrogen during energy restriction.
    c. aerobic exercise performance during energy balance and weight loss.
- To determine if initial plasma amino acid concentrations (or their change) are correlated with loss of body protein during energy restriction.

Hypotheses

H_o: There will be no difference in fasting plasma amino acid concentrations, apparent nitrogen balance and aerobic exercise performance during energy balance and energy restriction whether 40 $g \cdot d^{-1}$ whey protein isolate or casein is consumed prior to and during weight loss.

H_o: There will be no effect of acute ingestion of 20 $g \cdot d^{-1}$ whey protein isolate or casein along with a formula diet on concentrations of plasma free amino acids.

H_o: There will be no correlation between initial plasma amino acid concentrations (or their change) and loss of body nitrogen during energy restriction.

Delimitations

- The subjects were trained cyclists or triathletes between the ages of 19 and 29 years.
- Subjects had not consumed any whey protein dietary supplements for at least one month prior to the study.
- Subjects were randomly placed into treatment groups.

- Subjects' VO_{2peak} was determined no more than 2 wk before the first endurance exercise test.
- Intensity of endurance exercise tests was 70%, 55% and 90% of subjects' VO_{2peak}.
- The independent variable for the casein group was supplementation with casein (40 $g \cdot d^{-1}$) for 17 d prior to energy restriction and during the 4 d energy restriction period. The independent variable for the whey group was supplementation with whey protein isolate (40 $g \cdot d^{-1}$) for 17 d prior to energy restriction and during the 4 d energy restriction period.
- The dependent measures were plasma amino acid concentrations, time-to-exhaustion on a cycle ergometer at 90% VO_{2peak}, urinary nitrogen, urinary creatinine, and whole blood and white blood cell glutathione.
- The energy restriction diet was a formula diet (Ensure®, Abbott Laboratories) consisting of 20 $kcal \cdot kg^{-1} \cdot d^{-1}$ for 4 d with 63.9% CHO, 14.1% PRO, 22% Fat.

Limitations

- Subjects were free living. Thus, self-reported dietary intake, supplement consumption and energy restriction protocol adherence cannot be verified.
- Results can only be applied to individuals of similar age, training status and gender.
- There was no control group in this study to determine the effect of energy restriction on performance, plasma amino acid concentrations, glutathione and nitrogen balance without supplementation.
- No biochemical assessments of nutritional status were performed on the subjects prior to the beginning of the experimental period.
- No analyses of plasma amino acids were performed prior to the beginning of the experimental period.
- Activity was not controlled during the experimental period.
- There was no familiarization with the exercise protocol prior to the beginning of the experimental period. Thus, a learning effect may partially account for differences in performance between tests.

Definitions and Symbols

CHO	Carbohydrate
PRO	Protein
Glutathione (GSH)	The major endogenously produced antioxidant, consists of the amino acids cysteine, glutamate and glycine.
Immunocal®	Whey protein isolate product. Whey protein processed without the high temperatures, low pH and/or mechanical stress of typical pasteurization procedures.
Whey protein	A milk protein making up approximately 20% of the protein in cow's milk, it consists of β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulin and lactoferrin, among other compounds. The watery portion remaining after the precipitation of caseins during the production of cheese. This portion of milk is much higher in cysteine content than the other major protein portion of milk, casein.
Casein	A nonsoluble milk protein making up about 80% of the protein in cow's milk, it consists of α, β and κ components. Casein is the main constituent of cheese and is used in many commercial applications.
VO_{2peak}	Peak oxygen uptake. The highest level of oxygen use by skeletal muscle, heart, and lungs during an incremental cycling or running exercise protocol to exhaustion. It is considered an indicator of aerobic or cardiorespiratory physical fitness.
Apparent Nitrogen Balance (NBAL)	The difference between dietary nitrogen and total urinary nitrogen and estimates of dermal and fecal nitrogen losses.
Lean body mass (LBM)	Mass of all body tissue components save adipose tissue.
Creatinine	Crystaline compound formed in the breakdown of creatine phosphate. Excreted in the urine.
Aerobic	In the presence of oxygen.

Catabolism	Breakdown of complex molecules into smaller molecules. Destructive metabolism.
Casein group (CAS)	The group of subjects (n = 10) who consumed 40 $g \cdot d^{-1}$ casein for 21 d.
Whey group (WHEY)	The group of subjects (n = 10) who consumed 40 $g \cdot d^{-1}$ whey protein isolate for 21 d.
Essential Amino Acids (EAA)	Amino acids that cannot be synthesized within the human body and so must be obtained through the diet.
Nonessential Amino Acids (NEAA)	Amino acids that can be synthesized within the human body from the skeletons of other amino acids.
Branched Chain Amino Acids (BCAA)	Leucine, isoleucine and valine
Body Cell Mass (BCM)	Metabolically active tissue. LBM minus extracellular mass.
Reactive Oxygen Species (ROS)	Often referred to as free radicals, these very reactive, unstable oxygen species containing one unpaired electron include hydrogen peroxide, hydroxyl radical and superoxide anion.
Buthionine-sulfoximine (BOS)	Chemical that acts to inhibit glutathione synthesis by inhibiting γ-Glutamylcysteine synthetase.
N-acetyl cysteine (NAC)	Intravenously or orally administered antioxidant compound, often used in acetaminophen overdose treatment.
(RM)ANOVA	(Repeated Measures) Analysis of Variance

Basic Assumptions

- All subjects gave a maximal effort during the exercise testing protocols (VO_{2peak} testing, exercise tests, and performance tests).
- Subjects had not consumed any whey protein dietary supplements for at least one month prior to the study.

- Subjects refrained from nutritional supplements and stimulatory substances during the experimental period.
- Subjects consumed their entire 40 $g \cdot d^{-1}$ supply of protein each day during the experimental period.
- Subjects consumed their entire supply of Ensure® and no other calories during the 4 d energy restriction period.
- Subjects fasted for 10 h prior to blood draws for determination of fasted plasma amino acid concentrations.
- Subjects consumed one can of Ensure® 1 h prior to all endurance exercise tests.
- Subjects collected all of their urine during the 4 d energy restriction period.
- Protein supplements were 100% digestible.
- Dermal nitrogen losses were equal to 5.5 mg $N \cdot kg^{-1} \cdot d^{-1}$.

CHAPTER 2: REVIEW OF LITERATURE

Introduction

Athletes undergoing energy restriction to lose weight may experience reduced physical performance (108, 112) and loss of lean body mass (110). Some individuals look to nutritional methods to counteract these effects. Some studies suggest that specific plasma amino acid (AA) patterns predict body protein loss during catabolic states. For example, several researchers have observed a correlation between low plasma cysteine and loss of lean tissue (e.g. 44, 118) and others have demonstrated a correlation between cysteine levels and loss of body cell mass (62). Cysteine, an AA present in large amounts in the whey component of milk, is thus thought to play a vital role in the regulation of body protein metabolism. Whey protein has been observed to increase plasma cysteine concentrations in certain populations at risk for lean tissue loss (41, 57), but the product has been inadequately investigated in healthy populations. The effect of whey protein isolate on nitrogen balance (NBAL) in athletes also remains controversial and warrants further study.

An additional potential benefit of whey protein consumption is increased availability of cysteine for synthesis of the antioxidant glutathione (GSH). Decreased availability of GSH hastened fatigue in rats (e.g. 65, 98) and limited research in humans suggests that a whey protein supplement is ergogenic for high intensity exercise (66).

Thus, dietary supplementation with whey protein isolate may be of particular interest to dieting athletes who would like to maintain performance and reduce loss of lean body mass.

Use of urinary nitrogen and creatinine as measures of nitrogen balance

Unlike lipids and carbohydrates, the body is unable to store excess protein to be accessed in times of need. All protein in the body is put to use in tissue, transport molecules, storage molecules, enzymes, hormones, antibodies or in other capacities, with between 30-50% being used in the composition of skeletal muscle. Furthermore, nearly all of the nitrogen present in the body is incorporated into proteins, in their various forms (43).

Whereas in animal studies direct determination of changes in body protein is possible through carcass analysis, monitoring changes in humans is much more

problematic, especially over short periods of time. One widely accepted method for the determination of total body protein loss or gain is measurement of NBAL.

In a healthy adult consuming a eucaloric diet with adequate protein, body protein catabolism and anabolism occur at approximately the same rate, resulting in a state of neutral NBAL. In this situation, the amount of nitrogen consumed equals the amount excreted through the urine, feces and skin. Individuals in negative NBAL may have inadequate protein or caloric intake, resulting in a rate of body protein catabolism exceeding that of anabolism. Negative NBAL is indicated by levels of excreted nitrogen greater than those of ingested nitrogen.

Estimated NBAL is calculated according to the following equation: estimated NBAL = (protein intake (g)/6.25) – (urinary nitrogen (g) + fecal nitrogen (g) + dermal loss (g)). Nitrogen in the urine is present mainly as urea. Other, minor, sources of urinary nitrogen include creatinine and ammonia. These sources account for between 90-95% of nitrogen excreted from the body (43). The technique accepted as most valid for the determination of total urinary nitrogen involves a sulfuric acid digestion followed by titration (Kjeldahl). When using a formula diet, fecal losses can be estimated using a digestibility value for the source of protein intake. Dermal losses, although variable and increased during periods of intense sweating (63), can be satisfactorily estimated using a value determined by Rand et al. (88) (5.5 mg $N \cdot kg\ BW^{-1} \cdot d^{-1}$ in temperate climates) through regression analyses of 14 NBAL studies. Thus, when the nitrogen content of a diet is known, NBAL can be estimated through analysis of total urinary, fecal, and dermal nitrogen loss.

Creatinine is excreted in the urine following the breakdown of creatine phosphate. As creatine phosphate is present almost exclusively in muscle (98%) (52), creatinine excretion is used as a measure of muscle mass when creatine concentration is assumed to be constant. The correlation between creatinine excretion and lean body mass was first elucidated in 1913 by Myers and Fine (80). More recent studies have shown strong correlations between urinary creatinine excretion and muscle mass (49) and lean body mass (36, 78). Owing to the variability of urinary creatinine measures and the fact that significant amounts of the precursor can be found in meat; a consistent, meat-free diet is recommended for subjects to be screened using this method (49).

Urinary creatinine may be used in the measurement of muscle loss, but also serves another role. As urinary creatinine excretion remains relatively constant from day to day in those consuming a meat-free diet (9), creatinine values can be used to assess the completeness of subject urine collection; large daily variations in urinary creatinine suggesting incomplete collection. As complete 24 h urine collection is of paramount importance to accurate estimation of NBAL, urinary creatinine concentrations are used to form a ratio of urinary nitrogen to urinary creatinine (N/Cr), enabling researchers to form complete NBAL estimates in the case of incomplete collections.

While other, less invasive, methods of measuring the substantial loss of lean body mass characteristic of long term energy restriction have been shown to be valid (e.g. Dual Energy X-ray Absorptiometry), short-term changes, while not ignorable, are modest and most accurately measured using a NBAL technique.

Caloric restriction increases loss of body nitrogen

Although reduction of adipose stores is the objective of any weight loss protocol, many studies have demonstrated a loss of lean tissue with negative NBAL during weight loss. For example, Stanko and colleagues (103) observed negative NBAL to similar degrees in women consuming isonitrogenous 4.2 $MJ \cdot d^{-1}$ and 2.1 $MJ \cdot d^{-1}$ diets over a period of 21 d. Additionally, Smith et al. (102) reported that 17 $kcal \cdot kg\ BW^{-1} \cdot d^{-1}$ and 1$g \cdot kg\ BW^{-1} \cdot d^{-1}$ protein was sufficient to reduce baseline NBAL by 114 $mmol \cdot d^{-1}$, to -196 $mmol \cdot d^{-1}$, after 6 d in healthy men and women who lost an average of 1.7 kg. Likewise, 7 d of between 18.7 and 20.9 $kcal \cdot kg\ BW^{-1} \cdot d^{-1}$ and 1 $g \cdot kg\ BW^{-1} \cdot d^{-1}$ protein caused a cumulative negative NBAL of -24.49 g in trained young males (100). It has been suggested, however, that caloric restriction below a certain percentage of estimated energy needs may be necessary to elicit measurable losses of body protein, as Velthuis-te Wierik et al. (108) did not observe losses in lean body mass as assessed with hydrostatic weighing in nonobese men after 10 wk of consuming 80% estimated caloric needs.

Several processes have been suggested as the cause of this undesired protein loss when the body is in a state of caloric deficit. These include: increased reliance on AA as fuel, reduced anabolism due to dietary AA deficiencies, reduced anabolism due to decreases in IGF-1 with or without subsequent resistance to hGH, decreases in liver

protein synthesis, decreased T_3, decreased insulin secretion due to insufficient carbohydrate intake, and increases in body protein catabolism due to increased gluconeogenesis and/or cortisol. Additionally, weight loss through energy-restriction-induced caloric deficit seems to result in greater losses of both body weight and lean tissue than weight loss via energy expenditure-induced deficit, perhaps due to dissimilar metabolic changes elicited by each method (73).

The macronutrient composition of an energy-restricted diet may influence the loss of lean body mass which occurs during a period of weight loss. In particular, the amount of protein in an energy-restricted diet is thought to affect NBAL. In a study by Piatti et al. (87), obese women (mean BMI = 37.0) were fed 800 kcal·d^{-1} for 21 d. One diet contained 60% CHO and 20% PRO (0.6-0.8 g·kg BW^{-1}), the other 26% CHO and 45% PRO (1.4-1.6 g·kg BW^{-1}). Both groups lost similar amounts of weight, though the group consuming 45% PRO maintained a positive NBAL (+2.09 g·d^{-1} relative to baseline) and did not lose fat free mass (FFM) (-1.40 kg, not significant relative to baseline), while the group consuming 20% PRO had a negative NBAL (-5.32 g·d^{-1} relative to baseline) and did lose FFM (-3.02 kg). Although loss of FFM was assessed using circumferences and skinfolds, techniques that involve substantial error and lack sufficient sensitivity to accurately estimate modest changes over a 3 wk period, the differing NBAL measures suggest the low protein group experienced decreased anabolism. However, Hendler and Bonde (47) did not observe differences in loss of lean mass (2.10 vs. 1.61 kg) or NBAL in obese subjects consuming very low calorie diets (VLCD) of either 41% or 95% protein for 3 wk.

Similar investigations into the macronutrient composition of a weight loss diet for athletes have been completed, also with conflicting results. Work with active individuals in our lab led Walberg et al. (110) to suggest in 1988 that athletes undergoing energy restriction may require additional protein to avoid negative NBAL. In this study, men engaged in short-term energy restriction experienced negative NBAL (-3.29 g N·d^{-1}), despite consuming the Recommended Dietary Allowance (RDA) for protein and engaging in a resistance training program. In contrast, however, Mourier et al. (79) observed that elite wrestlers consuming between 24.4 and 31.6 kcal·kg^{-1}·d^{-1}, with or

without protein supplementation to 2 $g \cdot kg^{-1} \cdot d^{-1}$ for 19 d during their season, all suffered similar loss of muscle mass as estimated by MRI scan.

Others have suggested that the carbohydrate content of a weight loss diet may impact loss of lean tissue, although findings have been inconsistent. Vaswani et al. (107) propose that carbohydrate may be of negative influence to dieting individuals, as obese women consuming 800 $kcal \cdot d^{-1}$ diets containing 35% PRO and 10g CHO, with or without an additional 60g CHO via glucose supplements, for 12 wk, exhibited a trend toward a more negative NBAL when receiving the additional carbohydrate (-41.1 g N vs. -87.1 g N). However, a study from our laboratory (111) found that obese women consuming a VLCD of 33% CHO lost more total body weight and had greater urinary nitrogen losses relative to women consuming a VLCD of 71% CHO. In contrast, Stanko et al. (103) found similar NBAL in obese women receiving 50 $g \cdot d^{-1}$ PRO and either 75 g or 175 g CHO over 21 d.

Although regular exercise has been shown to increase muscle mass, caloric deficit may interfere with this effect. In a study reported by Keim et al. (58) and Van Loan et al. (106), for 12 wk overweight women consumed 50% of their estimated daily energy needs while performing aerobic exercise 6 $d \cdot wk^{-1}$ (D+E), or performed the exercise protocol while consuming a normal diet (E). While both groups experienced weight loss, D+E experienced more than twice the weight loss per week of E (1.1 $kg \cdot wk^{-1}$ vs. 0.5 $kg \cdot wk^{-1}$), with one-third of their weight loss coming from lean body mass, while E's weight loss was made up of only 14% lean body mass. D+E also exhibited an overall negative NBAL during the experimental period. However, through analysis with bioelectrical impedance, the researchers determined that this nitrogen loss did not come from muscle tissue. In addition, a study conducted by Zachwieja et al. (117) elicited average weight loss of 1.29 kg and trends toward increased nitrogen excretion ($p = 0.089$) and loss of lean body mass as measured by DEXA (-0.84 kg, $p = 0.093$) during 2 wk of caloric restriction at 750 kcal below regular daily energy intake in fit men and women who performed treadmill exercise to elicit a caloric expenditure of 500 kcal daily. Similarly, Walberg et al. (110) reported that short-term caloric restriction for weight loss in men leads to loss of lean tissue, as measured by NBAL (-3.29 $g \cdot d^{-1}$), despite consuming the RDA for protein and engaging in a resistance training program.

Conclusive research has shown both moderate and severe energy restriction of both short and extended duration to elicit negative NBAL. However, researchers have yet to agree on the ideal macronutrient composition of an energy restriction diet, or a dietary supplement, to lessen the undesirable loss of body nitrogen. While some suggest protein supplementation to be beneficial in reducing lean tissue loss, the available data is not unanimous nor does it provide for recommendation of a specific protein supplement. Several authors have suggested that characteristics of whey protein isolate, such as its AA composition, make it an appealing candidate for use in the evasion of nitrogen loss during caloric restriction, yet the utility of the supplement in this capacity remains untested.

Caloric restriction reduces performance

Keys et al. (59), Neiman et al. (81) and Webster et al. (114) have demonstrated significant reductions in aerobic performance during semi-starvation, fasting and energy restriction with dehydration, respectively. Reductions in physical performance often also coincide with less rigid energy restriction practices (112). Although several causes have been proposed for these performance declines, such as micronutrient deficiencies, glycogen depletion, muscle tissue damage, altered muscle enzyme activity and negative mood states, neither a definitive mechanism for the reduced performance nor a weight loss technique consistently avoiding the reductions have been elucidated.

Performance declines have been noted in both obese and healthy populations restricting energy intake. Phinney et al. (85) observed decreased aerobic capacity in obese subjects consuming a ketogenic diet for 4 wk. Similarly, obese women consuming a VLCD for 4 wk suffered decreased VO_{2peak} in $l \cdot kg^{-1}$ despite regular aerobic training during the energy restriction period (111). In a study of healthy men undergoing 10 wk of caloric restriction at 80% normal energy intake, time to exhaustion on a cycling test at the completion of the experimental period significantly decreased despite the subjects not exhibiting measurable losses of protein (108). McMurray et al. (74) reported decreased total and average power in collegiate wrestlers on a Wingate Cycling Test after 7 d of caloric restriction at 65% estimated energy needs, but performance on an 8 min run at 85% VO_{2peak} did not suffer.

However, not all studies are in agreement. In separate case studies, Maffulli (70) and Widerman and Hagan (115) reported improved VO_{2peak} ($mL \cdot kg^{-1} \cdot min^{-1}$) in two wrestlers who lost 8% body weight via energy restriction over 3 wk, and unchanged VO_{2peak} in another wrestler observed over two months of dieting. Koutedakis et al. (64) did not observe a significant change in the VO_{2peak} ($L \cdot min^{-1}$) of elite oarswomen following 2 months of dieting, but reported a significant increase in VO_{2peak} after 4 months. As mentioned above, although Zachwieja et al. (117) were able to elicit weight loss and increased nitrogen excretion with their energy restriction design, performance measures, including Wingate Test, 5-mile run time and leg and shoulder press 1-RM were maintained or improved in the dieting subjects. The varied results of these studies suggest that duration of caloric restriction may be of influence on decreases in aerobic performance.

Several mechanisms have been hypothesized to explain the causes of aerobic exercise performance declines experienced with short-term energy restriction. Dehydration has conclusively been shown to negatively impact performance, as well as other physiological markers (e.g. 114). When hydration status is not a factor, the carbohydrate content of an energy restricted diet has been suggested to influence performance due to its impact on glycogen stores. Bogardus et al. (12), for example, observed a significant decrease in aerobic performance in obese women after just 1 wk on a 830 $kcal \cdot d^{-1}$ diet with 1% CHO while women consuming an isocaloric diet containing 36% CHO did not exhibit performance declines. Others (111), however, have witnessed nearly identical improvements in performance measures while consuming VLCD either high or low in carbohydrate.

A loss of body protein, skeletal muscle protein in particular, due to either increased catabolism, reduced synthesis, or both, is also hypothesized to contribute to performance losses during energy deficit. Our research group has reported impaired muscular function in power athletes suffering from protein loss during energy restriction (110, 113). These consequences on muscle tissue are also thought to possibly negatively influence endurance exercise performance during energy deficit, especially during short periods of dieting which may not allow sufficient time for metabolic or mechanical adaptations to occur that might help compensate for decreased muscular or metabolic

function. Unfortunately, few studies of the effects of short-term energy restriction on aerobic performance, not involving dehydration, have been done, so many hypothesized factors to reduced performance remain unconfirmed.

As the studies above demonstrate, a reduction in performance measures during energy restriction is an often encountered problem. Athletes often make efforts to reduce performance declines, as well as loss of lean tissue, during caloric restriction despite the lack of consensus as to the cause of these drawbacks. Some research has been conducted to determine the value of dietary supplements for this purpose. Research from one laboratory found that supplementation with either arginine (113) or creatine (92) did not prevent performance declines during energy restriction, nor did they significantly influence body protein loss. Others have suggested that other AA (82) or supplements (79) may help maintain aerobic performance during energy restriction, but direct studies of performance in endurance athletes are lacking. Demonstration that a nutritional supplement reduces loss of body nitrogen and performance declines during weight loss would be attractive to many of these athletes.

Whey protein supplementation may affect performance and body composition

Several studies have investigated the effect of whey protein supplementation on athletic performance and/or body composition, although results are equivocal. Burke and colleagues (20) investigated whey protein supplementation (1.2 $g \cdot kg^{-1} \cdot d^{-1}$) compared to an isoenergetic carbohydrate supplement in men engaged in a 6 wk weight-training program, finding increases in lean tissue (2.3 kg) and knee extension torque (7%) when compared to subjects involved in resistance exercise alone (0.9 kg and 5% torque increase). The conclusions that can be drawn from this study are limited, however, in that the whey protein supplement was not compared to any other type of protein supplement and may indicate only that a protein supplement is superior to carbohydrate during resistance training. In addition, although the difference was not significant, according to 3 d diet records, those subjects consuming whey protein consumed an average of 2.1 $g \cdot kg^{-1} \cdot d^{-1}$ PRO through their normal diet in addition to the supplement, while control intake averaged only 1.2 $g \cdot kg^{-1} \cdot d^{-1}$.

In contrast, Agin et al. (2) did not find that supplementation with 1 $g \cdot kg^{-1} \cdot d^{-1}$ whey protein for 14 wk during resistance training had any more benefit on gains in body cell mass or skeletal muscle mass than resistance training alone in HIV-infected women. Data from another study (4) showed inferior gains in lean body mass in trained individuals undergoing aerobic and resistance training and consuming 20 $g \cdot d^{-1}$ whey protein, relative to those receiving an equal dosage of bovine colostrum. There were no differences between groups in this study in both aerobic and anaerobic exercise performance measures.

Several other recent studies have used whey protein as a "placebo" in the investigation of bovine colostrum or other proteins, with variable results (e.g. 17, 19, 51). Hofman and colleagues (51) provided male and female field hockey players with 60 $g \cdot d^{-1}$ whey or colostrum for 8 wk. While both groups exhibited improved 5 x 10 m sprint performance at the end of the supplementation period, those receiving colostrum exhibited a modestly greater increase. In a study by Buckley et al. (19), 30 active men consumed 60 $g \cdot d^{-1}$ whey and bovine colostrum for 8 wk. Both groups increased peak running speed – a measure the experimenters claimed was equivalent to peak power – at both 4 and 8 wk of supplementation, but there was no difference between groups.

Demling and DeSanti (30) observed that supplementation with whey protein hydrolysate to achieve a protein intake of 1.5 $g \cdot kg^{-1} \cdot d^{-1}$ while consuming a calorie restricted diet of 80% estimated needs and participating in a 4 $d \cdot wk^{-1}$ weight training program resulted in greater gains in strength and lean tissue and loss of adipose tissue, than training and dieting without the supplement. Other subjects in this study, receiving a casein protein hydrolysate in amounts identical to subjects receiving the whey supplement, demonstrated even greater gains in performance and body composition measures, however.

In opposition to the findings of the above study, Lands et al. (66) reported decreased fat mass in healthy subjects consuming a whey protein supplement compared to no change for those consuming a casein supplement for 3 months. However, the subjects consuming whey also reported increased physical activity relative to placebo during the experimental period, clouding the relationship between the two observations.

Limited research suggests that supplementing the diets of persons participating in resistance training with whey protein may aid in gains of strength and lean tissue, at least relative to not supplementing at all. However, it is unclear what properties of whey protein are responsible for the benefits observed in these trials. Additionally confounding conclusions is the fact that differences in the processing of various whey and other protein supplements leave them with heterogeneous characteristics and biological effects, meaning that the whey protein supplement studied in one experiment may not be equivalent to a whey protein used in another study. Moreover, the effect of whey protein supplementation in athletes participating in endurance exercise has yet to be studied. These gaps in the scientific knowledge beg investigation into the effect of whey protein isolate on endurance athletes, with focus on elucidating a mechanism for the supplement's action.

Plasma amino acids and body protein loss

Several studies (32, 44, 118) have shown low plasma cysteine levels in individuals suffering from catabolic diseases such as breast cancer, HIV, and Crohn's disease.

Researchers have noted the apparent association of plasma cysteine levels with lean tissue catabolism and suggested that cysteine in the blood indirectly exerts a regulatory effect on body protein catabolism via the amino acid's direct relationship with hepatic urea production which enables it to impact muscle glutamine concentrations. When cysteine is broken down into sulfate in the liver, protons are released as byproducts. Increased proton concentrations result in greater degradation of bicarbonate into water and carbon dioxide. As bicarbonate is also a substrate for carbamoylphosphate synthesis, production of the latter is hindered, succeeded by a drop in urea production. This in turn results in higher concentrations of ammonia, retarding the breakdown of glutamate into α-ketoglutarate. The resulting increase in glutamate concentrations, in addition to the presence of greater levels of ammonia, favors the synthesis of glutamine. Through this mechanism, maintenance of plasma glutamine stores is enhanced. Greater stores of glutamine, which can be converted to other forms of energy and the homeostasis of which has been suggested to maintain an anabolic/catabolic balance in multiple tissues

and organs, are thought to result in a reduced reliance on the catabolization of lean tissue occurring with the efflux of glutamine from skeletal muscle during periods of stress (31).

This anabolic/catabolic balance is hypothesized to be maintained by the "postabsorptive glutamate/cystine shuttle." Droge and Holm (31) attribute decreases in plasma cysteine in wasting diseases and overtraining to dysregulation of this shuttle, which directs protein catabolism in the muscle, initiating cysteine release in order to maintain plasma levels of the AA as cysteine is broken down in the liver to abate urea production in the postabsorptive state (31). Dysregulation of the shuttle is thought to occur via substantial increases in plasma urea concentrations caused by several possible conditions, including states eliciting a cytokine response and increased Cori cycle activity. Increases in urea, such as those seen in cachexia (68), lead to increased lean tissue catabolism in an attempt to obtain free cysteine as a substrate for proton generation in order to limit the enhanced urea production, according to this theory. Thus, in addition to the diseases mentioned above, exercise and caloric restriction (states both eliciting increased Cori cyle activity) meet the criteria for inclusion as conditions possessing the ability to produce low levels of plasma cysteine.

Reductions in plasma glutamine levels have coincided with plasma cysteine decreases in multiple studies of chronic diseases associated with lean tissue loss (32, 44, 118). Increased urea production is also characteristic of these conditions (66). These changes, including decreased plasma glutamine (83) and increased serum urea concentrations (29), have also been seen in athletes undergoing long-term endurance exercise. While these studies of athletes did not measure plasma cysteine levels, the observations are interesting considering the hypothesized mechanism for cysteine's regulatory effect on body protein catabolism.

Data from one study of healthy persons exhibited correlations between low plasma cysteine levels and muscle mass loss during an exercise program. Kinscherf et al. (62) reported a correlation between both low baseline plasma cysteine and glutamine levels and body cell mass (BCM) loss in healthy individuals undergoing a 4 wk weight- and interval-training program. Subjects having low baseline cysteine levels also exhibited a negative correlation between their increased, post-exercise program cysteine levels, and the exhibited loss of BCM, suggesting that lean tissue had been catabolized, at

least in part, in order to increase plasma cysteine concentrations. This theory was strengthened by the finding that supplementation of the subjects with 400 mg $3x \cdot wk^{-1}$ exogenous cysteine via N-acetylcysteine (NAC) curtailed BCM loss predicted by low plasma cysteine levels. The researchers suggest that the high level of glycolytic activity present in subjects undergoing anaerobic training is similar to the activity present in cachexia, and that this activity retards glutamate transport causing muscle catabolism. The authors also reported that, in addition to low cysteine levels, high levels of plasma glutamate, and low levels of glutamine, were risk factors for BCM loss in healthy subjects, in accordance with the proposed glutamate/cystine shuttle mechanism for body protein maintenance.

Although the correlation of baseline cysteine levels with BCM loss in the above study suggests that plasma cysteine can be predictive of lean tissue loss in healthy individuals as well as those suffering from disease, loss of BCM was also found to correlate with baseline levels of glutamate, glutamine and arginine, and with changes in concentrations of glycine, tyrosine and phenylalanine. Additionally, the researchers hypothesized that the change in BCM might correlate with levels of glutathione (GSH), a compound synthesized from cysteine, but they did not validate this theory by measuring levels of the antioxidant, highlighting the need for investigation of the association of this compound with changes in lean tissue.

A follow-up to the study by Kinscherf et al. was conducted in which researchers measured AA exchange rates in healthy and diseased subjects. In healthy individuals, release of the AA cysteine, tyrosine and phenylalanine from body protein, the latter two of which can be used as muscle catabolism markers, was negatively correlated with plasma levels of thirteen AA. However, cysteine was not one of the AA with which release rates were found to correlate (44). In agreement with the hypothesis that the system responsible for AA exchange is dysregulated in wasting diseases, however, cancer patients did not exhibit any correlation between plasma AA levels and exchange rates.

The results of the above-reviewed studies suggest that supplementing plasma concentrations of cysteine and glutamine may be of benefit to individuals in danger of losing LBM. The critical role for glutamine in anabolic/catabolic balance has been known for some time, with various methods being attempted to boost stores of the AA.

As direct supplementation with glutamine is problematic, due to its oxidation within the intestinal tract and absorption by other organs, it has been hypothesized that exogenous cysteine, available in a protein supplement, may help abate the loss of lean tissue in individuals at risk for this occurrence. This is still conjecture, however, and has yet to be directly investigated.

Cysteine and glutamine are not the only AA plasma concentrations of which have been hypothesized to affect body protein metabolism. The branched chain AA (BCAA) – isoleucine, leucine and valine – have received much attention for their hypothesized role in regulating muscle protein metabolism. These three AA have been observed to increase in concentration in the plasma during catabolic states (101). Branched chain AA are metabolized mainly in the skeletal muscle and can be used as a fuel source for this tissue during exercise. These facts have led many to hypothesize that high circulating levels of BCAA may inhibit lean tissue proteolysis for the release of BCAA for use as energy in individuals undergoing exercise or experiencing caloric deficit.

Branched chain AA are also suggested to stimulate protein synthesis and/or retard proteolysis. The BCAA leucine is believed to function both as a substrate and signal in protein synthesis (3). Additionally, BCAA have been seen to improve NBAL in humans suffering from catabolic states (e.g. 38, 53). Supplementation with BCAA has also been associated with more positive NBAL than supplementation with other AA in healthy subjects undergoing bed rest (104). However, other studies have shown no improvement in NBAL with BCAA administration in ill patients (25, 96).

Some researchers have observed that supplementing with BCAA prevented decreases in plasma glutamine elicited by long-term exercise (83) and research in rats suggests that BCAA may increase endogenous glutamine synthesis (86). This implicates BCAA in roles similar to that proposed for cysteine by Droge and Holm (31), as a substrate used to regulate plasma free glutamine concentrations. In a study comparing whey and casein protein consumption on plasma AA concentrations in healthy men and women, Hall et al. (46) observed significantly greater concentrations of free valine, isoleucine, leucine, threonine and total AA in the plasma, relative to baseline, beginning at 45 min and continuing until 180 min following the consumption of a meal high in whey content versus a casein meal. Unfortunately, it is unclear if concentrations of

glutamine and cysteine were measured in this study. These findings suggest another possible mechanism through which whey protein supplementation may improve NBAL in dieting athletes.

While plasma free AA have been studied for decades, much about the significance of levels of individual AA remains not well understood. Significant research supports a role for plasma cysteine in the regulation of lean tissue catabolism in various populations. The influence of plasma concentrations of cysteine and glutamine on NBAL in dieting athletes is still unknown. The lack of knowledge demands investigation into the role of these AA on loss of nitrogen and the ability of a protein supplement to influence free AA levels in an athletic population.

Characteristics of whey and casein proteins

In addition to its carbohydrate, fat and mineral constituents, milk consists of 3.2% protein. Of this, the vast majority is casein and whey proteins. Casein components (α, β and κ in cow's milk) make up approximately 80% of the protein portion of milk. Whey protein, consisting of β-lactoglobulin, α-lactalbumin, immunoglobulins, serum albumin and lactoferrin, makes up most of the additional 20% (99).

Despite shared origins and status as complete proteins, the two proteins have many different characteristics. For example, while whey is readily soluble in water, casein is an insoluble protein – the protein found in the "curd" formed in the manufacture of cheese. Casein is also used to produce paint, adhesives and plastics. The whey separated from curds in cheese manufacture has found fewer industrial uses and is in turn abundantly available as a waste product.

Since antiquity, milk has been recognized as possessing health-benefiting properties. Science has recently allowed for the isolation of the sources of many of these activities. When whole casein and whey proteins are hydrolyzed during digestion, the products are peptides and free AA. Many of these peptides have been found to have biological activities and the concentrations of individual AA within the plasma affected by protein ingestion are also thought to influence physiological processes. The activities of peptides derived from milk protein may be elicited within the gut or following absorption and transport, in peripheral tissues and circulation, and include influencing

hormone secretion, nutrient uptake and blood pressure (23). Additionally, both protein components of milk have been much researched for their opioid and immunological effects. Casein has been most intensely investigated for its possession of opioid agonist (76) and antagonist (99) activities, while research into the immuno-enhancing properties of milk peptides have generally focused on the whey component (although colostrum, secreted immediately before milk, has been widely studied for its obvious immunological effects) (116).

In humans, the abilities of these two milk proteins to affect NBAL and body protein metabolism have mainly been compared using the proteins as components of formula feedings for pre-term infants and hospitalized patients (e.g. 27, 91). These studies have generally involved mixtures of more than one protein source and/or protein hydrolysates, which often do not produce biological effects identical to those of whole proteins. In healthy individuals, the superiority of whey or casein to the other for stimulating lean tissue accretion is debated due to the differing AA compositions and speeds of digestion of the proteins.

Fruhbeck (39) addressed the different AA compositions of the two proteins and pointed out the greater amounts of BCAA and lesser amounts of glucagon release-stimulating AA in whey relative to casein. This author suggested that catabolism due to glucagon is lower and anabolism stimulated by BCAA greater after whey consumption than following ingestion of casein, theoretically making whey a superior protein supplement for those trying to avoid loss of lean tissue. In contrast to the hypothesis of Frubeck, however, Dangin et al. (26), highlight the fact that whey is considered a "fast" protein, while casein is regarded as "slow," as the latter coagulates in the stomach increasing emptying time. These researchers put forth that the "slow" protein casein is preferable to whey for avoiding overall loss of protein, as the former creates a longer postprandial hyperaminoacidemia, reducing protein breakdown. Data from their study in which a mixed-meal containing whey protein or casein was fed to healthy young adults, suggests a greater postprandial leucine balance in those consuming casein. However, conclusions concerning these results are hindered by the fact that the treatments were not isonitrogenous.

Forming the basis of Fruhbeck's hypotheses concerning whey and casein are the differences in their raw AA compositions. Whey is generally found to contain more alanine, cysteine and BCAA, and less tyrosine and arginine, than casein. Although, due to varied hydrolysis, absorption and uptake and processing in the liver and intestine, the AA composition of a dietary protein is often not reflected proportionately in tissue AA concentrations, the amounts of certain AA provided to the body by a protein source often affect tissue levels, and may influence hormone release, skeletal muscle metabolism, cellular signaling, protein and peptide synthesis, and osmoregulation, among other activities. The possible influence of concentrations of specific free AA on NBAL in catabolic states has already been reviewed above.

Numerous studies have been performed investigating the physiological consequences of milk and milk protein consumption. While it has been repeatedly shown that whey and casein produce varying physiological effects in the relative short-term following digestion, the relevance of these different effects in adults is debated. Furthermore, few studies have investigated the effects of chronic ingestion of a whey or casein supplement in healthy adults undergoing energy restriction. Additionally, it is widely agreed that milk proteins and the products of their digestion possess bioactive properties, some of which have been described above. Several of these bioactivities have been repeatedly demonstrated and are beginning to be understood. Other consequences of milk protein consumption are anecdotal or without mechanistic support. Undoubtedly, still more physiology-modulating aspects of milk proteins have yet to be discovered. This has not prevented the lay nutritional supplement consumer from being bombarded with claims of the physiological benefits of consuming various milk protein products. Specific studies producing findings applicable to the athlete wishing to improve performance and body composition, are lacking. More study of whey and casein, two widely-available, commonly-originating, yet very different, proteins is clearly warranted in athletes, specifically if and how either of the proteins is able to lessen loss of body nitrogen or improve aerobic performance and/or antioxidant status.

Dietary intake, exercise and plasma amino acid profile

The dynamic characteristics of free AA within the body have been the subjects of research for many years. The plasma AA profile has been of particular focus, despite making up only 0.5% of the entire pool of AA, and levels of AA in the plasma have been found to be affected by diet, physiological state, transport capacity, the metabolism of other tissues, age and gender (1). The effect on plasma glutamine and/or cysteine concentrations specifically, with varied diets, training status and/or nutritional supplementation, either acutely or chronically, is lacking, however. Additionally, even when researchers have investigated the influence of these variables on glutamine, cysteine has rarely been reported due to difficulties in its analysis. Despite deficiencies in the available knowledge, limited research suggests that supplementation with a whey protein supplement may enhance and sustain plasma levels of cysteine and glutamine, but this hypothesis is as-of-yet untested in healthy persons undergoing energy-restriction.

As reviewed by Abumrad and Miller (1), dietary intake has significant effects on plasma AA concentrations. Hormones released in the presence or absence of food have varying effects on the entire AA pool, as well as on the concentrations of individual AA and types of AA. More specifically, the protein source, its AA composition, the timing of ingestion and whether it is consumed with other macronutrients, all influence appearance of plasma free AA (1). The varied hydrolysis of dietary proteins before or during digestion, absorption of peptides and free AA by the intestine and use of these compounds within digestive tissue may result in changes in plasma AA concentrations that differ from protein to protein and often do not mirror dietary composition.

The effects of different diets and meal timings on plasma cysteine and glutamine have been investigated in several studies. Scriver et al. (97) measured plasma AA in healthy adults over 270 min while fasting and after one and two meals. Although the levels of individual AA varied widely through the experimental period, of particular note was the consistency in values for cysteine and glutamine during the course of the experiment. Although cysteine concentrations tended to be higher in the fasted state, they were not significantly so (mean of both groups combined, 61$\mu mol \cdot L^{-1}$). These levels also changed little in the immediate hour following a meal. While glutamine levels were significantly higher in fasted subjects (726 $\mu mol \cdot L^{-1}$ vs. 586 $\mu mol \cdot L^{-1}$), levels of fasted

and non-fasted subjects remained parallel with little change during the course of measurements, also without noticeable change in the measurement made following lunch. Bergstrom et al. (7) observed decreased plasma concentrations of all essential AA save histidine, after a protein-free meal, but reported increases in most essential AA in the hours following a meal containing 50 g serum albumin. Levels of cysteine and glutamine did not change following the protein-free meal, but cysteine concentrations were significantly increased relative to baseline at both 1 and 3 h post-ingestion of the meal containing albumin (a minor component of whey protein which is responsible for a large fraction of its cysteine content). Cysteine levels returned to pre-meal levels 5 h postprandially.

Similar to the above studies, Forslund et al. (37) observed little change in plasma glutamine levels of samples taken every 30 min for 24 h following a meal and during exercise in subjects consuming diets containing either 1 $g \cdot kg^{-1} \cdot d^{-1}$ or 2.5 $g \cdot kg^{-1} \cdot d^{-1}$ milk protein for 7 d. Of note in this study is the inverse relationship of plasma glutamine concentrations with level of protein intake. Subjects receiving a diet of 1 $g \cdot kg^{-1} \cdot d^{-1}$ protein exhibited fasted glutamine concentrations of 712 μM while those consuming additional protein had much lower levels (403 μM). The differences between groups were maintained both during exercise and postprandially. They are also in agreement with data from another study in which subjects consuming 0.1, 0.8 and 2.2 $g \cdot kg^{-1} \cdot d^{-1}$ protein for 3 wk exhibited postabsorptive plasma glutamine concentrations of 616 μM, 492 μM and 411 μM, respectively (71), suggesting that chronic high protein intake leads to reduced levels of free glutamine in the plasma. However, Cahill et al. (21) observed no changes in postabsorptive plasma glutamine concentrations in subjects consuming diets either high in carbohydrate or high in protein.

A limited number of studies have attempted to influence plasma cysteine and/or glutamine via dietary supplements or changes. An early study by Sanchez and Swendseid (95) reported that 3 d of a cysteine and methionine free diet was sufficient to reduce plasma cysteine concentrations in rats. Results of a study by Bounous and Kongshavn (15) showed no difference in plasma cysteine levels compared to baseline in rats receiving lactalbumin, a major component of whey protein and responsible for a significant portion of its cysteine content, or casein, for 2 wk. All other measured AA

that differed in content by more than 10% in the two groups, however, exhibited significant differences in concentration. Working with humans, Kashyap et al. (57) compared plasma AA in premature infants fed an 18% whey formula or 60% whey formula. While differences between treatments did not reach significance, mean plasma cysteine concentrations were approximately 20% higher in subjects receiving the whey-predominant formula following several weeks of consumption. Subsequently, Gazzaniga and colleagues (42) administered total parenteral nutrition with and without supplemental cysteine to hospitalized adults for an average of 11.4 d. Those receiving the supplemented formula had significantly higher serum cysteine/cystine levels than those patients receiving unsupplemented formula. Subjects receiving additional cysteine also tended to have a more positive NBAL than those not administered the extra cysteine ($p = 0.07$) and total cysteine/cystine and cystine correlated with NBAL in both groups.

Some researchers have looked at the influence of energy deficit on plasma AA profile. While starvation generally increases essential AA concentrations concomitant with decreases in nonessential AA, the opposite tends to occur in low calorie diets deficient in protein (69). A study by Gatti et al. (40) of obese adults consuming 500 $kcal \cdot d^{-1}$ supports this generalization, as levels of threonine, valine, isoleucine and lysine, but also glycine, increased during the VLCD. Fisler et al. (34) reported similar findings as concentrations of several essential AA decreased and nonessential AA increased in obese subjects consuming a VLCD supplemented to 1.3 $g \cdot kg^{-1} \cdot d^{-1}$ with either soy or collagen protein for 40 d. While glutamine levels were not reported, there was no effect of group or energy restriction on levels of cysteine despite 13-fold differences in cysteine content between the two proteins.

Other researchers have examined plasma AA in subjects participating in acute bouts of exercise, finding varying effects depending on length of bout. Some have reported insignificant changes in plasma cysteine and glutamine following 3 h of running (52) or 20 min of cycling and a short cycling bout to exhaustion (18). Longer events, however, have produced significant changes. Decombaz et al. (29) reported that a 100-km run caused plasma cysteine to increase by approximately 70% from a pre-race level of 36 $\mu mol \cdot L^{-1}$. Following this same race, glutamine concentrations decreased by approximately 55 $\mu mol \cdot L^{-1}$, from 352 $\mu mol \cdot L^{-1}$ before the competition. Twenty-four h

later, glutamine was still around 28 $\mu mol \cdot L^{-1}$ below baseline. Notably, the baseline glutamine levels of these subjects were similar to those of chronically fatigued elite athletes in another study (61). In contrast, Lehmann et al. (67) measured plasma AA concentrations in athletes competing in the Colmar ultra triathon. Post-race serum glutamine levels were unchanged in these subjects although they were much more variable than baseline levels which had been 500 $\mu mol \cdot L^{-1}$. Similar to the study by Decombaz and colleagues, however, cysteine levels had increased significantly from baseline following the race.

In one of the few studies of training state on plasma AA profile, Kinsgbury et al. (61) measured plasma AA of elite (mainly endurance) athletes training for the Olympic Games. Although the authors did not report concentrations of cysteine, they observed concentrations within the "normal" range for the 19 AA reported in these subjects. They also investigated elite athletes suffering from fatigue and reported decreased levels of multiple AA, including glutamine, in these subjects. Those athletes suffering from plasma glutamine concentrations below 450 μM were counseled to increase dietary protein intake and returned for AA analysis 3 wk later at which time glutamine concentrations had significantly increased (378 μM vs. 592 μM).

Research has shown that individuals participating in exercise experience changes in the makeup of the plasma AA pool and that training status possibly influences baseline levels. Changes in the plasma free AA pool are also seen in those consuming low calorie diets. As has been discussed previously, there is evidence to suggest that plasma levels of cysteine and glutamine may influence loss of lean tissue and that interventions to increase circulating concentrations of these AA may be beneficial. Dietary composition clearly influences levels of circulating free AA in the plasma, but appearance is seldom proportional to amount ingested. Limited data supports the ability of whey protein to enhance plasma cysteine levels. Unfortunately, few researchers have reported specific data on plasma cysteine concentrations in their studies of the impact of diet and exercise on AA levels. Furthermore, many studies have only involved measures of fasted AA levels. These may not be the only levels of interest when considering regulation of proteolysis, as different proteins, including whey and casein, produce peak concentrations of individual AA at varying times following ingestion. How changes in AA levels due to

caloric restriction in trained athletes are affected by unhydrolyzed whey or casein protein supplementation and the physiological consequences of any effects remain in question.

Increase in GSH may delay fatigue

Levels of reactive oxygen species (ROS) beyond the capacity of in vivo reductive compounds can lead to tissue damage (28), and intense physical activity has been shown to be capable of inducing such levels of ROS (54). GSH is a critical component in the body's ROS scavenging machinery and cysteine is a limiting agent in the biosynthesis of this antioxidant.

During aerobic exercise, increased oxygen consumption leads to an increase in levels of ROS in the blood (72). Accelerated muscular fatigue may be one result of increases in ROS. Indeed, Barclay and Hansel (6) reported an increased rate of fatigue in isolated rodent muscle exposed to free radicals. Sen et al. (98) reported a 50% reduction in time-to-exhaustion on a treadmill in rats treated with BSO, a chemical that inhibits GSH synthesis. Similarly, Kramer and colleagues (65) observed a reduction in swim performance of rats given diethyl maleate, another GSH synthesis inhibitor. These studies suggest that GSH has a role in the delay of fatigue during aerobic exercise.

One of the few studies examining the relationship between GSH and muscular fatigue in humans was conducted by Reid and colleagues (90) employing the compound NAC, an agent shown to increase plasma levels of GSH (16). In their experiments, fatigue in electrically stimulated tibialis anterior muscles was retarded by intravenous administration of NAC. However, the high incidence of adverse effects and danger of overdose associated with NAC relegate it to use solely as a clinical agent and disqualify its use as a supplement. In addition, not all studies have shown performance benefits with NAC administration. A recent study (75) infused NAC intravenously into non-trained individuals performing intermittent exercise. While NAC infusion was found to increase levels of GSH in the blood, this did not affect performance on a cycling test to fatigue at 130% VO_{2peak} following three 45 s bouts at the same intensity.

The common dietary supplement, whey protein isolate, has been shown in several studies to increase plasma levels of GSH (14). The processing of this supplement is believed to be critical to its ability to increase levels of GSH. In contrast to that prepared

through typical, high-temperature pasteurization procedures, whey protein which is filtered to remove bacteria is touted to maintain its cysteine content in the oxidized form of the AA, cystine, and in glutamylcysteine peptides. These forms avoid the breakdown experienced by free cysteine within the GI tract due to the latter's potential toxicity. Upon cellular entry, cystine may be reduced to cysteine, the form of the AA used in the synthesis of GSH.

Lands et al. (66) showed that healthy men and women who consumed 20 g of a whey protein isolate supplement daily for 3 months had a 35% increase in lymphocyte GSH and a 13% increase in peak power and total work performed during a maximal cycling bout of 30 s. While such a significant increase in performance measures is encouraging, the exercise test employed was atypical given the proposed mechanism of the supplement. Research does not support 30 s maximal cycling test performance to be limited by antioxidant capacity. As whey protein isolate is proposed to delay fatigue via increased GSH stores to combat oxidative stress, an endurance performance test that has been shown to induce oxidative stress would be a more appropriate tool to analyze the hypothesis. Nevertheless, the above studies suggest a possible benefit of a whey protein supplement on GSH and delay of fatigue, but further investigation of this hypothesis is needed.

Summary

Low levels of plasma cysteine have been observed to be predictive of the body protein catabolism characteristic of various disease states. A similar correlation has been observed in healthy persons participating in an exercise program, and enhancing plasma cysteine levels succeeded in preventing loss of BCM in these subjects. While voluntary caloric restriction for weight loss has been shown to cause a loss of lean body mass as measured by NBAL, and certain diets have been found to increase plasma cysteine levels, the ability of a whey protein supplement to retard the loss of lean tissue via increases in plasma cysteine concentration has not been investigated. Whey protein supplementation has been associated with increases in glutathione in athletic populations and these increases are thought to improve aerobic exercise performance by improving antioxidant capacity, reducing free radicals and in turn delaying fatigue. The impact of the

supplement on exercise performance in dieting individuals is unknown. Athletes who are interested in weight loss, but who would also like to maintain lean body mass and performance, will be interested in the prospect of avoiding these negative side effects of dieting with a common dietary supplement.

CHAPTER 3:

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Effect of whey or casein on plasma amino acids and nitrogen balance in athletes

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ABSTRACT

PURPOSE: To compare the effects of whey and casein on plasma AA, nitrogen balance (NBAL), glutathione and performance in dieting athletes. **METHODS:** Twenty cyclists consumed 40 $g \cdot d^{-1}$ whey (WHEY) or casein (CAS) for 3 wk. On d 18 – 21 subjects restricted intake to 20 $kcal \cdot kg^{-1} \cdot d^{-1}$ plus protein supplement. Apparent NBAL was estimated on d 18 – 21 while postabsorptive and postprandial plasma AA were measured on d 14 and 21. On d 1, 15 and 22 subjects performed an exercise performance test and provided blood for glutathione analyses. **RESULTS:** Both groups experienced similar negative NBAL (CAS = -19.7 ± 1.4 g, WHEY = -21.4 ± 2.7 g) while dieting. There were trends towards reduced performance during energy restriction ($p = 0.073$) and an interaction of group with day ($p = 0.072$). There were effects of state (postabsorptive = 34.5 ± 2.4 μM, postprandial = 37.1 ± 3.0 μM; $p = 0.038$) and time (d 14 = 33.8 ± 2.2 μM, d 21 = 37.8 ± 3.2 μM; $p = 0.008$) on plasma cysteine. There was an interaction of state and time on glutamine ($p = 0.002$), as levels increased 1.3% from postabsorptive to postprandial measurements on d 14, but decreased 4.2% on d 21. The absolute change in postabsorptive cysteine from d 14 to d 21correlated with NBAL ($r = 0.766$, $p = 0.01$) in CAS but not in WHEY. Plasma glutamine did not correlate with NBAL in either group. **CONCLUSION:** Protein source did not affect NBAL, performance, glutathione, or plasma cysteine and glutamine. Dieting increased plasma cysteine and reduced postprandial glutamine. Changes in plasma cysteine may be related to NBAL during energy restriction.

Keywords: cysteine, glutamine, glutathione, Immunocal®

Introduction

As research has shown athletic performance to negatively correlate with fat mass (63), athletes often engage in energy restriction in order to reduce body fat and improve athletic performance (22, 44). Many athletes consume nutritional supplements with the same intent (25). Other work, however, has detected a negative impact on athletic performance during and immediately following an energy-restriction period (59, 61). Science has yet to determine an ideal nutritional supplement for consumption during energy restriction with which to avoid performance losses.

Another unwanted consequence of energy restriction is the loss of body nitrogen. Numerous studies have produced data showing a negative nitrogen balance (NBAL) in subjects undergoing caloric restriction (53 – 55, 60). Increased protein consumption during energy restriction is thought to improve NBAL (46), however, various nitrogen sources may not have identical effects on NBAL (47).

Plasma amino acid (AA) profiles may be affected by different diets or protein sources (1). The relationship between AA content of specific protein sources and postabsorptive and postprandial plasma AA concentrations are unclear, as are any relationships these AA levels may have to NBAL and performance in athletes undergoing energy restriction. Some researchers have suggested that plasma levels of cysteine and glutamine may affect loss of body cell mass in those suffering from wasting diseases (19) and in healthy persons undergoing exercise (35). Whey protein is particularly rich in the AA cysteine. The ability of a whey protein supplement to affect plasma free AA levels and any effects of supplementation on performance during caloric restriction remains untested.

During aerobic exercise, increased oxygen consumption leads to an increase in levels of reactive oxygen species (ROS) in the blood (40). Glutathione (GSH) is a critical component in the body's ROS scavenging machinery and cysteine is a limiting agent in the biosynthesis of this antioxidant. As it is relatively high in cysteine content, the common dietary supplement, whey protein isolate, has been investigated in several supplementation studies and found to increase levels of GSH in erythrocytes and white blood cells (WBC) (9, 37). Intense physical activity has been shown to be capable of inducing levels of ROS beyond the capacity of in vivo reductive compounds (33).

Accelerated muscular fatigue may be one result of increases in ROS (5, 36, 49, 52). Such levels of ROS can also lead to tissue damage (17).

Researchers have observed improved athletic performance coinciding with increased WBC GSH in subjects consuming a whey protein supplement (37). Thus, increased GSH levels resulting from whey protein supplementation may be beneficial for athletes desiring to improve performance. Additionally, others have hypothesized that increased GSH stores may protect against lean tissue loss that may occur in association with tissue damage associated with high levels of ROS and during times of stress (35).

Any relationship between GSH and lean tissue loss has yet to be shown, the ergogenicity of improved GSH levels remains questionable, evidence of the catabolism modulating effects of enhanced plasma cysteine levels is limited and the ability of a common whey supplement to produce the preceding conditions has been insufficiently tested. This study investigated the effect of whey protein isolate and casein on plasma AA, NBAL, GSH and performance in trained, male cyclists undergoing energy restriction.

Methods

Subjects

Twenty males between the ages of 19 and 29 years were included in this experimental study. Most subjects were well-trained, cycling for an average of nearly 6 h or over 100 miles per week, with most actively competing in collegiate cycling and/or triathlon contests. Subjects had not consumed any whey protein-containing nutritional supplements for at least one month prior to the beginning of the experimental period and refrained from taking any nutritional supplements during the experimental period. Subjects completed a health history questionnaire to ensure they met the criteria of the American College of Sports Medicine (ACSM) for "low risk" for exercise participation and testing. Additional exclusionary criteria related to the low calorie diet and supplement consumption included eating disorders and food allergies. Subjects gave their informed consent and the study was approved by the Institutional Review Board.

Subject Pre-Testing

Baseline measurements of body composition and aerobic fitness were performed on all subjects no more than 2 wk prior to the beginning of the experimental study. Body composition was determined using a three-site skin fold equation derived by Jackson and Pollock (32). The same trained technician performed all skin fold measurements at the abdomen, suprailliac and tricep.

Peak oxygen consumption (VO_{2peak}) was determined for all subjects using a graded exercise test on a Sensormedics 800 cycle ergometer (Sensormedics, Yorba Linda, CA). Prior to VO_{2peak} testing, subjects were familiarized with exercise on the ergometer at a constant pedaling rate while breathing through a mouthpiece and breathing valve. The test began at a low intensity, 100 watts, with subjects pedaling at a preferred cadence, and increased 15 watts every 30 s until the subject could no longer maintain 60 rpm or reached volitional exhaustion. Continuous indirect calorimetry was performed during the test using a Sensormedics Vmax 229 metabolic cart system (Sensormedics). Resistance, perceived effort and oxygen consumption were recorded every 30 s. The wattage found to elicit 55%, 70% and 90% of VO_{2peak} during the peak oxygen consumption test was calculated and used as resistance during the submaximal exercise and time-to-exhaustion tests.

Subjects had measurements of body weight taken during pre-testing, as well as prior to submaximal exercise testing on d 1, 15 and 22 of the experimental period.

Submaximal Exercise and Time-to-Exhaustion Tests

Subjects reported to the lab on the mornings of d 1, 15 and 22 of the experimental period, 1 h after consuming a standardized breakfast of one can (237 mL/250 kcal) of Ensure® formula diet (Abbott Laboratories, Abbott Park, IL) (63.9% CHO, 14.1% PRO, 22% Fat) and no other food or drink save water. Subjects were positioned on the cycle ergometer and allowed to warm-up with a resistance of 100 watts for 5 min. Immediately following the warm-up period, subjects began a submaximal exercise bout entailing 7 min at a resistance that elicted 70% VO_{2peak} followed by 38 min at a resistance associated

with 55% VO_{2peak}. Oxygen consumption was measured using a Sensormedics Vmax 229 metabolic cart once during the 70% phase and twice during the 55% phase.

At the completion of the 45 min submaximal exercise test, subjects stopped pedaling for approximately 1 min while blood samples were taken. Subjects then immediately began a timed test to exhaustion at 90% VO_{2peak} until they could no longer maintain 60 rpm or until volitional exhaustion.

Protein Supplementation and Caloric Restriction

Subjects were randomized to either the whey protein isolate (WHEY, n = 10) or casein group (CAS, n = 10). Both experimenters and subjects were blinded to group membership. From d 1 to d 17 of the experimental period, in addition to their normal diets, subjects consumed $40 g \cdot d^{-1}$ of protein corresponding to group placement. Amino acid composition of each protein is shown in Table 1. Subjects were given 1 wk supplies of protein on d 1 and d 8 that consisted of 14 bags each containing 20 g of protein which had been weighed out by the experimenters, and a 3 d supply (6 bags) on d 15, and instructed to consume one bag of protein in the morning and one in the evening daily. On d 1, subjects received instructions concerning method of protein consumption, which included a warning to avoid blending the protein supplements or mixing the supplements in hot foods or liquids. Subjects were also instructed to consume the supplements no more than 30 min following mixing of the protein in a food or beverage. At the end of each week, subjects returned a compliance form detailing patterns of protein supplement consumption.

On d 18, 19, 20 and 21 subjects consumed $40 g \cdot d^{-1}$ of protein in addition to 20 $kcal \cdot kg^{-1} \cdot d^{-1}$ of a formula diet (Ensure®). Subjects came to the lab each morning and received a 1 d supply of formula diet and protein.

Urine Samples and Analysis

Subjects completed 24 h urine collections during each of the 4 d of the energy restriction period. Samples were collected in 1L polypropylene bottles containing 2 mL 50% hydrochloric acid to ensure that ammonia did not become volatile. Collections began with the second void of the day and were brought to the lab after the first void of

the next morning. The samples were mixed well and total volume was recorded. Aliquots were frozen for later analysis of nitrogen and creatinine.

Urine samples were thawed at room temperature and creatinine concentration was analyzed in duplicate spectrophotometrically according to directions from a commercially available kit (Creatinine Procedure No. 0400, Stanbio Laboratory, Boerne, TX). Duplicates with greater than 10% difference were reanalyzed. Urinary nitrogen was analyzed in duplicate using the Kjeldahl technique. Duplicates with greater than 7% difference were reanalyzed.

Apparent NBAL was calculated for each day of the energy restriction period separately. Dietary nitrogen was calculated using the manufacturer's data for nitrogen content of the formula diet, while nitrogen contents of the protein supplements were analyzed by the experimenters using the Kjeldahl technique and found to contain 13% nitrogen by mass. Fecal nitrogen was estimated using the protein digestibility value for the formula diet provided by the manufacturer (Abbott Laboratories) -- 94.2% digested, 5.8% lost in feces. Given the milk source of the protein supplements, they were assumed to have essentially 100% digestibility and thus not contribute to fecal nitrogen. Dermal nitrogen loss was estimated using the value established by Rand et al. (48), of 5.5 mg $N \cdot kg^{-1} \cdot d^{-1}$ for temperate climates.

Blood Collection and Analysis

On the mornings of d 14 and 21, following an overnight fast of at least 10 h, subjects reported to the lab and blood samples were taken from an arm vein immediately prior to and 2 h following consumption of one can of Ensure® mixed with 20 g of protein supplement, to determine the effects of caloric restriction on postabsorptive and postprandial plasma AA concentrations. Blood samples were immediately spun at 1500 X g for 15 min at 4ºC. Plasma was immediately removed after centrifugation and thoroughly mixed with 25.6 $mg \cdot L^{-1}$ norleucine in methanol in a 1:2 ratio. The plasma-methanol mixture was capped, refrigerated overnight at 4ºC and then spun at 27000 X g for 20 min at 4ºC. The supernatant was decanted, purged with nitrogen gas and stored at -20ºC for later analysis of plasma AA. Plasma AA were analyzed in duplicate by AA analyzer (PICO.TAG, Waters Association, Milford, MA).

Blood samples for the analysis of GSH were collected on d 1, 15 and 22 immediately before beginning the submaximal exercise test. White blood cells were separated from one sample of whole blood using Vacutainer Cell Preparation Tubes (Vacutainer CPT, Becton Dickinson and Co., Franklin Lakes, NJ). After washing with PBS, WBC were resuspended in 200 µl PBS and counted using a Coulter Counter (Beckman Coulter, Inc., Fullerton, CA). Samples of both whole blood and WBC samples were frozen at -70º C for future analysis. Both whole blood and WBC GSH were measured using a commercially available spectrophotometric assay (GSH/GSSG-412 assay, OxisResearch, Portland, OR). Whole blood was analyzed for total protein using a detergent free protein assay (Bio-Rad Laboratories, Hercules, CA) in order to report GSH per mg protein.

Statistical Procedures

Baseline characteristics and cumulative NBAL of the two groups were compared using independent samples t-tests and paired samples t-tests, respectively. Nitrogen balance, performance and GSH data were analyzed using the repeated measures ANOVA function within the SPSS statistical package with group and day as main factors. Plasma AA were analyzed using repeated measures ANOVA with a 2x2x2 factorial design with group (WHEY and CAS), day (d 14 and d 21) and state (postabsorptive and postprandial) as main factors. Post-hoc pairwise t-tests, with Bonferroni correction for multiple comparisons where appropriate, were performed on analyses with significant omnibus F in ANOVA analyses. Correlations between AA concentrations and total NBAL are represented by Pearson's correlation coefficients and were arrived at using bivariate correlation analyses with a two-tailed test of significance. A p value of less than or equal to 0.05 was determined *a priori* as the standard for significance.

Results

There were no significant differences between groups for age, weight, body fat percentage, VO_{2peak}, and training volume at baseline (Table 2), nor were there differences between groups for body weight at any point during the experimental period. There were also no significant differences between groups for absolute change or percent change in

body weight between any two laboratory visits. Both groups experienced significant weight loss as a result of the energy restriction protocol ($p < 0.001$) with a mean body weight loss for CAS of 2.9 ± 0.3 kg (3.9%) and 2.5 ± 0.4 kg (3.4%) for WHEY.

Adherence to Protocol

There was 100% reported consumption of protein supplement in all subjects. Subjects were also queried daily during energy restriction as to total collection of urine and consumption of formula diet. All subjects reported total urine collection. One subject failed to consume one can (237 mL/250 kcal) of formula diet on the first day of the energy restriction period. Appropriate alterations were made in the calculation of this subject's estimated NBAL for d 18.

There were no reports of any adverse effects from either of the protein supplements.

Exercise Performance

There was a trend toward decreased performance with energy restriction in both groups ($p = 0.073$). There was also a trend toward an interaction of group with day on performance measures ($p = 0.072$) as CAS performance was improved on d 15 relative to d 1, while performance by WHEY was essential unchanged. It should be noted, though, that CAS and WHEY had n of 9 and 7, respectively, for the comparisons, due to the exclusion of data for four subjects who did not adhere to the experimental protocol during the timed test to exhaustion during at least one of the tests, due to equipment malfunction or experimenter error.

Nitrogen Balance

No urine collections exhibited greater than 25% variation in 24 h creatinine excretion from the mean of an individual, so no urine collections were excluded from analyses. There was a significant main effect of day on NBAL ($p = 0.001$) with subjects experiencing significantly less negative NBAL on d 19 and 20 than on d 18 and 21 (Figure 2). There was also a significant main effect of day on urinary nitrogen excretion per gram urinary creatinine ($p = 0.025$), with a higher value for both groups combined on

d 18 (10.21 ± 0.22) than on d 19 and 21 of the energy restriction period (d 19 = 9.48 ± 0.20; d 20 = 9.79 ± 0.28; d 21 = 9.67 ± 0.24). There was no effect of group on NBAL on any one day of the caloric restriction period (Figure 2), nor was cumulative 4 d NBAL different between groups (CAS = -19.7 ± 1.4 g, WHEY = -21.4 ± 2.7 g).

Plasma Amino Acids

No measurements of plasma AA were made prior to the beginning of protein supplementation. Mean concentrations of plasma AA for each group at each of four time points during supplementation are displayed in Table 3.

Of consequence to the original hypothesis, there were significant main effects of state (PA = 46.5 ± 3.2 μM, PP = 50.0 ± 4.1 μM; $p = 0.038$) and day (d 14 = 45.5 ± 2.9 μM, d 21 = 50.9 ± 4.3 μM; $p = 0.008$) on plasma concentrations of cysteine, without a significant difference in the effect of group. There was a significant interaction between day and state on levels of glutamine ($p = 0.002$), as levels increased 1.3% (+8.5 μM) from the postabsorptive to postprandial state on d 14, but decreased 4.2% (-28.4 μM) on d 21. The absolute and percent change in postabsorptive plasma cysteine from d 14 to d 21 exhibited a positive correlation with total NBAL (absolute – $r = 0.766$, $p = 0.01$; percent – $r = 0.769$, $p = 0.009$) in CAS but not in WHEY ($p = 0.922$) or when all subjects were combined ($p = 0.553$). There was no correlation between NBAL and absolute concentrations of glutamine or changes in plasma concentrations of glutamine observed in either group.

Data concerning plasma concentrations of other AA were also analyzed and means representing significant main effects are shown in Table 4. There was a main effect of group on plasma levels of tyrosine ($p = 0.006$) with greater concentrations exhibited by CAS. There was a main effect of state on the plasma concentrations of glutamic acid ($p = 0.017$), glycine ($p = 0.029$), isoleucine ($p < 0.001$), leucine ($p < 0.001$), methionine ($p < 0.001$), ornithine ($p < 0.001$), proline ($p < 0.001$), taurine ($p = 0.010$), tyrosine ($p < 0.001$) and valine ($p < 0.001$). Each of the preceding AA increased in concentration in the plasma 2 h postprandially, except glycine and taurine, which decreased in concentration. Day exhibited a significant main effect on plasma concentrations of several AA, with citruline ($p = 0.025$) increasing in concentration and

glutamic acid ($p = 0.005$), ornithine ($p = 0.008$) and proline ($p = 0.002$) decreasing during energy restriction. There was a main effect of state on plasma branched chain amino acids (BCAA) ($p < 0.001$), essential amino acids (EAA) ($p < 0.001$), non-essential amino acids (NEAA) ($p < 0.001$), and sulfur amino acids (methionine and cysteine combined) ($p = 0.001$), with concentrations increasing from the postabsorptive to the postprandial state, but there was no effect of group. Sulfur amino acids exhibited a significant main effect of day ($p = 0.008$), increasing during energy restriction, while NEAA exhibited a trend toward an effect of day on concentrations ($p = 0.069$).

There was a significant interaction between day and state on levels of threonine ($p = 0.004$), arginine ($p = 0.006$) and histidine ($p = 0.049$) in the plasma. Threonine concentrations increased by 14.8% (27.5 ± 4.7 μM) from the postabsorptive to the postprandial state on d 14, but increased only 7.6% (14.4 ± 4.1 μM) on d 21. Concentrations of arginine increased 10.0% (8.6 ± 2.7 μM) following meal consumption on d 14, but increased only 2.6% (2.2 ± 2.9 μM) during energy restriction. Mean postabsorptive levels of plasma histidine were 5.5% greater (d 14 PA – 84.7 ± 2.3 μM; d 21 PA – 89.3 ± 2.7 μM) during energy restriction than during energy balance.

There were significant interactions between state and group in the plasma concentrations of histidine ($p = 0.010$), phenylalanine ($p = 0.014$) and tryptophan ($p = 0.009$). Specifically, CAS experienced an increase in plasma histidine concentrations of 6.2 % (5.5 ± 2.2 μM) and WHEY a decrease of -4.6% (3.9 ± 1.9 μM) from the postabsorptive to the postprandial state on both days combined. Meal consumption caused the concentration of phenylalanine in CAS to increase 7.1 ± 2.0 μM (+9.6%) to 80.6 μM, a concentration significantly greater than that of WHEY, which decreased 2.3 ± 1.8 μM (-3.3%) to 66.6 μM. In contrast to histidine and phenylalanine, levels of tryptophan increased to a greater extent in WHEY following meal consumption (52.1%; 35.0 ± 3.7 μM) than in CAS (22.5%; 16.0 ± 3.5 μM).

There was also a significant interaction between day and group on levels of histidine ($p = 0.022$). Concentrations of plasma histidine in CAS decreased slightly (0.3 ± 1.5 μM; -0.3%) with energy restriction, from 91.6 ± 2.0 μM to 91.4 ± 2.7 μM, while levels in WHEY were significantly lower on d 14 and increased 4.8 ± 1.7 μM (+5.9%) from 80.9 ± 2.3 μM to 85.6 ± 2.1 μM during the diet period.

There were also three-way interactions between day, state and group on the plasma concentrations of serine ($p = 0.021$), alpha-aminobutyric acid ($p = 0.007$), asparagine ($p = 0.001$) and lysine ($p = 0.014$). Plasma serine, lysine and asparagine levels in WHEY experienced a different response to meal consumption during energy restriction relative to during energy balance, while CAS had a similar response at both times. Levels of serine increased 13.5% (12.0 ± 4.0 μM) and 14.5% (15.2 ± 3.1 μM) in WHEY and CAS, respectively, following meal consumption on d 14. On d 21, however, mean plasma serine increased 12.1% from 108.5 μM in the postabsorptive state to 121.6 μM postprandially in CAS, while levels in WHEY decreased 2.2% from 103.7 μM to 101.5 μM. Plasma concentrations of free lysine in CAS increased 28.9% (48.7 ± 8.5 μM) and 26.7% (46.8 ± 10.2 μM) following meal consumption on d 14 and d21, respectively. WHEY, however, experienced a reduced response during energy restriction, displaying an increase of 21.8% (35.7 ± 9.2 μM) over postabsorptive levels on d 21, whereas a 42.3% (66.5 ± 10.3 μM) increase was experienced on d 14. A similar, attenuated response of plasma asparagine levels to meal consumption during energy restriction was observed in WHEY, as concentrations increased 18.7% (17.1 ± 5.3 μM) on d 14, but only 5.3% (5.2 ± 4.7 μM) on d 21. CAS, in contrast, displayed similar increases of 13.8% (13.7 ± 3.7 μM) and 16.4% (16.4 ± 4.3 μM) on d 14 and d 21, respectively. Concentrations of alpha-aminobutyric acid were much higher in both groups during energy restriction, both postabsorptively and postprandially, but the effect of meal consumption on levels of the AA differed between groups during this time, although not during energy balance. On d 14, levels increased 1.7% (0.5 ± 1.4 μM) and 3.6% (1.1 ± 2.2 μM) following meal consumption in WHEY and CAS, respectively, but on d 21, levels of alpha-amiobutyric acid in CAS increased by 2.4% (1.0 ± 2.4 μM) from 41.6 μM while concentrations in WHEY decreased by 12.0% (5.9 ± 1.5 μM) from 48.8 μM.

In addition, the concentrations of several AA and changes in these concentrations exhibited correlations with cumulative NBAL (Table 5). These include negative correlations between NBAL and postabsorptive levels of tryptophan ($r = -0.637$, $p = 0.048$) and ornithine ($r = -0.704$, $p = 0.023$), and postprandial levels of proline ($r = -0.726$, $p = 0.017$) and ornithine ($r = -0.718$, $p = 0.019$) on d 14 in CAS. A positive correlation between 4 d cumulative NBAL and postabsorptive and postprandial levels of

valine ($r = 0.679$, $p = 0.031$ and $r = 0.703$, $p = 0.023$, respectively) and leucine ($r = 0.741$, $p = 0.014$ and $r = 0.662$, $p = 0.037$), and postprandial levels of tryptophan ($r = 0.675$, $p = 0.032$) was seen in WHEY on d 14. A negative correlation between cumulative NBAL and postabsorptive phenylalanine concentration ($r = -0.686$, $p = 0.028$) was observed on d 21 in WHEY, while positive correlations with NBAL were detected in postprandial levels of serine ($r = 0.653$, $p = 0.041$), histidine ($r = 0.639$, $p = 0.047$), isoleucine ($r = 0.633$, $p = 0.050$), leucine ($r = 0.698$, $p = 0.025$) and tryptophan ($r = 0.698$, $p = 0.025$) on d 21 in this group.

When both groups were combined, correlations between NBAL and plasma AA concentration were observed in d 14 postabsorptive levels of valine ($r = 0.490$, $p = 0.028$) and leucine ($r = 0.458$, $p = 0.042$), d 21 postabsorptive phenylalanine concentrations ($r = -0.551$, $p = 0.012$), and d 21 postprandial histidine levels ($r = 0.451$, $p = 0.046$). Changes in postabsorptive phenylalanine ($r = -0.445$, $p = 0.049$) and postprandial citruline ($r = 0.484$, $p = 0.031$) and ornithine ($r = 0.517$, $p = 0.020$) also exhibited correlations with NBAL in both groups combined.

Correlations between absolute BCAA and cumulative NBAL were observed in the postabsorptive ($r = 0.699$, $p = 0.024$) and postprandial states ($r = 0.674$, $p = 0.033$) on d 14 and postprandial state on d 21 ($r = 0.644$, $p = 0.044$) in WHEY, but no correlations between levels of this group of AA were present in CAS.

In WHEY, the absolute changes in postprandial plasma serine and citruline from d 14 to d 21 exhibited a positive correlation with total NBAL ($r = 0.667$, $p = 0.035$ and $r = 0.844$, $p = 0.002$, respectively), whereas levels of postabsorptive valine demonstrated a negative correlation ($r = -0.665$, $p = 0.036$). CAS exhibited positive correlations between cumulative NBAL and absolute change in ornithine concentrations in the postabsorptive ($r = 0.827$, $p = 0.003$) and postprandial ($r = 0.815$, $p = 0.004$) states, as well as a significant positive correlation between changes in postabsorptive tryptophan and cumulative NBAL ($r = 0.694$, $p = 0.026$).

Correlational analyses of percentage change in AA concentration and cumulative NBAL were also performed with significant correlations mirroring those noted for absolute changes above. These include a positive correlation between percent change in postprandial ornithine and NBAL ($r = 0.513$, $p = 0.021$) and a positive correlation

between percent change in postprandial citruline and cumulative NBAL ($r = -0.486$, $p = 0.03$) in both groups combined. In addition, WHEY exhibited a posisitve correlation between percent change in postabsorptive citruline and NBAL ($r = 0.870$, $p = 0.001$) and a negative correlation between percent change in postabsorptive valine concentration and NBAL ($r = -0.665$, $p = 0.036$). CAS exhibited positive correlations between cumulative NBAL and percentage change in ornithine concentrations in the postabsorptive ($r = 0.767$, $p = 0.01$) and postprandial ($r = 0.746$, $p = 0.013$) states, as well as a significant positive correlation between changes in postabsorptive tryptophan and cumulative NBAL ($r = 0.641$, $p = 0.046$).

Amino acid levels were not found to correlate in any way with performance measures.

Glutathione

Values for whole blood and WBC GSH can be seen in Table 6. White blood cell GSH increased 40% ($p = 0.016$) over d 1 and d 15 levels following energy restriction in both groups combined, but there was no significant difference between treatments.

There was a mean increase in whole blood GSH of 9.9% from d 1 (1316 ± 32 μmol/mg protein) to d 15 (1446 ± 52 μmol/mg protein) in WHEY, but it was not significant. There was no change in whole blood GSH in CAS. Energy restriction resulted in a non-significant 5% decrease in whole blood GSH for both groups combined.

Neither whole blood nor WBC GSH concentrations were found to correlate with performance measures or NBAL.

Discussion

The results of this study indicate that 4 d of energy restriction at 20 $kcal \cdot kg^{-1} \cdot d^{-1}$ resulted in negative NBAL and increased WBC GSH in young, well-trained male athletes, but that neither whey nor casein is superior to the other in reducing loss of body nitrogen or affecting GSH stores. However, these proteins were found to have differing effects on postabsorptive and postprandial levels of multiple AA both during energy balance and deficit in this population. Correlations between levels of several AA and

NBAL suggest that plasma concentrations of free AA and groups of AA may influence loss of nitrogen during energy restriction.

Nitrogen Balance

Numerous studies have demonstrated a loss of lean tissue with negative NBAL during weight loss (46, 54, 55). Thus, our observations of negative nitrogen balance throughout the energy restriction period are not surprising. Subjects in our study received between 1.09 to 1.30 $g \cdot kg^{-1} \cdot d^{-1}$ protein (mean = 1.20 $g \cdot kg^{-1} \cdot d^{-1}$) during the energy restriction period and lost an average of 2.7 kg. Both groups in our study experienced negative daily and cumulative NBAL. The protein intake of our subjects was about 1.5 times the Dietary Reference Intake (DRI) (31) for protein and slightly greater than that of subjects in a study conducted by Smith et al. (53), of trained young males consuming between 18.7 and 20.9 $kcal \cdot kg\ BW^{-1} \cdot d^{-1}$ and 1 $g \cdot kg\ BW^{-1} \cdot d^{-1}$ PRO for 7 d. This protein consumption caused a cumulative negative NBAL of -24.49 g in these subjects. It appears as if recommended levels of protein may not be suitable for athletes undergoing weight loss. Indeed, Walberg et al. (60) suggested that athletes undergoing energy restriction may require additional protein to avoid negative NBAL. In that study, men engaged in short-term energy restriction experienced negative NBAL (-3.29 g $N \cdot d^{-1}$), despite consuming the DRI for protein and engaging in a resistance training program. It is possible that with even greater daily protein consumption during energy restriction our subjects may have maintained positive, or suffered less negative, NBAL. It also seems likely that the amount and type of activity during energy restriction impact nitrogen loss (58, 65), and that training volume should be considered when planning a nitrogen-sparing weight loss protocol for athletes. Variations in day-by-day training may partially account for the more negative NBAL on d 21, relative to d 20 in our subjects.

Several previous studies have compared the effects of whey and casein proteins on body composition with conflicting results. Demling and DeSanti (18) observed that supplementation with whey protein hydrolysate while dieting resulted in greater gains in lean tissue and loss of adipose tissue than resistance training and dieting without the supplement. However, other subjects in this study receiving a casein protein hydrolysate in amounts identical to subjects receiving the whey supplement demonstrated even

greater gains in body composition measures. In opposition to the findings of the above study, Lands et al. (37) reported decreased fat mass in healthy subjects consuming a whey protein supplement, compared to no change for those consuming a casein supplement for 3 months. However, the subjects consuming whey also reported increased aerobic activity relative to placebo during the experimental period, clouding the proposed relationship. Our research has not quelled any debate surrounding any protein-sparing properties of these two proteins. As both groups in our study suffered similar negative NBAL, our results fail to conclusively demonstrate the superiority of a single protein supplement with which to limit nitrogen loss during energy deficit.

Despite the identical origins of whey and casein, these proteins possess greatly differing characteristics. These include AA composition, rate of digestion, impact on gastric emptying and bioactivity of peptides contained within the proteins (8, 26, 39). Owing to these significant differences, the nutritional values of the two proteins have been often compared. Many of the comparisons have involved growth of human infants and young animals or healing of diseased or burned individuals, however, with few comparisons involving healthy adults or athletes. Many other researchers have used hydrolysates of whey and/or casein, preparations which may differ in biological activity and value to whole proteins, in their investigations

To our knowledge, this is the first study to compare the effects of whey protein isolate and casein on NBAL in trained adults undergoing energy restriction. Our data suggest that the proteins do not differ in regard to their effects on NBAL over a brief period in this population. Fruhbeck (23) suggests that the amount of glucagon release-stimulating AA in whey is less than in casein, and thusly that catabolism due to glucagon is lower after whey consumption than following ingestion of casein, theoretically making whey a superior protein for those trying to avoid proteolysis. Concentrations of insulin and glucagon were not measured in the present study, so the above statement could not be verified in our subjects. In contrast to the hypothesis of Frubeck, however, Dangin et al. (16) put forth that the "slow" protein casein is preferable to whey for protein accretion, as the former creates a longer postprandial hyperaminoacidemia. Data from their study of a mixed-meal containing whey protein or casein, suggest a greater postprandial leucine balance in those consuming casein. As our analyses of postprandial AA concentrations

included only a single time point, 2 h following meal consumption, we are also unable to compare the effect of the two supplements over an extended postprandial period. The lack of a control group does not allow us to determine if any differences of protein effect on hormones or duration of hyperaminoacidemia are manifested in reduced nitrogen loss. The lack of a difference in NBAL between groups suggests that any benefit from consumption of one of the investigated proteins is no greater than the effects of the other for maintaining body nitrogen during energy restriction.

Kinscherf et al. (35) observed correlations between plasma cysteine concentrations and losses of BCM in persons engaged in anaerobic exercise. Beyond theorizing that plasma cysteine levels may influence proteolysis, these researchers hypothesized that because of the status of cysteine as the limiting factor in GSH synthesis, that the antioxidant may also be related to loss of lean tissue. We investigated the above possibility in our population, but found no correlations between whole blood or WBC GSH and NBAL in our subjects.

Our study has not resolved the questions surrounding the optimal composition of a protein supplement to avoid loss of body nitrogen during weight loss. A similar amount of body nitrogen was lost by both groups, giving no indication that either whey or casein supplementation at 40 $g \cdot d^{-1}$ is superior to the other for preventing loss of nitrogen during short-term energy restriction in this population.

Performance

Whey protein has been oft tested in recent years for its purported ergogenic effects. Many of these studies have involved resistance training (14) or tests of peak power or work capacity (15, 29). Several recent studies have used whey protein as a "placebo" in the investigation of bovine colostrum or other proteins, with variable results (12, 13, 29). The whey protein isolate administered to WHEY in our study, Immunocal®, is touted to increase stores of the antioxidant GSH due to its unique processing which leaves glutamylcysteine peptides and cystine residues intact, a characteristic apparently absent from most other available whey supplements. Lands et al. (37) investigated this supplement, reporting a 35% increase in lymphocyte GSH and a 13% increase in peak power and total work performed during a maximal cycling bout of 30 s in healthy men

and women who consumed 20 $g \cdot d^{-1}$ for 3 months. Although the exercise test employed by the group was similar to those in other investigations of whey supplements, the test was atypical given the proposed mechanism of this particular supplement. Research does not support 30 s maximal cycling test performance to be limited by antioxidant capacity. As the whey protein in question is proposed to delay fatigue via increased GSH stores to combat oxidative stress, an endurance performance test that has been shown to induce oxidative stress would be a more appropriate tool to analyze the hypothesis. It was our intent, by employing the exercise protocol described in this article, to investigate whether this particular whey protein supplement might delay fatigue in calorically restricted individuals.

While energy restriction can be expected to influence performance on the test used in this study, the trend toward an interaction of group with day is more difficult to explain. This observation is likely attributable to remarkably different changes in performance in WHEY and CAS between d 1 and 15, with CAS increasing mean time-to-exhaustion by 92.6 ± 24.0 s and WHEY decreasing mean time-to-exhaustion by 12.9 ± 35.8 s. It must be noted though, that the CAS and WHEY had n of 9 and 7 for the comparisons, due to the exclusion of data for four subjects who did not adhere to the experimental protocol during the timed test to exhaustion during at least one of the tests. It is possible that supplementation with CAS during this period is responsible for the trend. If this is the case, the variables measured in this study do not provide clues as to the mechanism behind the trend toward improved performance, as neither NBAL nor GSH were affected by group. It is also possible that owing to a lack of controls on subject diet and training prior to the beginning of the experimental period, by chance CAS subjects were in a more fatigued state than WHEY subjects on d 1 of the experimental period, accounting for the lower mean time-to-exhaustion. The conclusions that can be made from these performance data are limited due to the fact that we failed to perform multiple performance trials prior to the experimental period in order to ensure the reliability of the test.

There was not a significant effect of whey protein isolate or casein on GSH levels. Although we did witness a directional increase in average WBC GSH of 13.5% in WHEY, it was not statistically significant. In the study by Lands et al. (37), WBC GSH

increased 35% after 3 months of supplementation at 20 $g \cdot d^{-1}$ with the same whey protein isolate used in our study. Although a pilot study performed in our laboratory in a small sample of a similar population (results not shown) suggested that 2 wk of 40 $g \cdot d^{-1}$ whey protein were sufficient to increase whole blood and WBC GSH, it is possible that a longer duration of supplementation is necessary to significantly affect GSH levels. It is notable, however, that the untrained subjects in the pilot study had baseline whole blood GSH concentrations 63% lower than subjects in the present study. It is possible that individuals who are aerobically well-trained may experience maximized GSH levels as a training adaptation (50), making GSH boosting interventions less effective, and avoiding any possible ergogenic effects experienced by less-trained populations. Recent studies have cast doubt on the preceeding statement, however, as Rush and Sandiford (51) did not find a relationship between levels of glutathione peroxidase and aerobic fitness. Additionally, in a study by Medved et al. (42), individuals performing a submaximal and time-to-exhaustion cycling protocol nearly identical to ours showed performance, although not improved during an infusion of N-acetylcysteine (NAC), an agent proven to increase plasma levels of GSH (11), to be correlated with VO_{2peak}. Notably, however, these studies did not involve weight loss or energy restriction.

As previously mentioned, Lands et al. (37) observed increased WBC GSH in nondieting individuals supplementing with whey protein while a similar increase was not observed in subjects receiving a casein supplement. In contrast, we did not see an effect of group on WBC GSH levels. We did, however, observe a 40% increase in WBC GSH in our subjects during energy restriction. We cannot assess whether the increase in WBC GSH in our subjects affects performance, however, as our study did not include a control group with unchanged WBC GSH stores against which to assess ergogenicity. The occurrence of increased WBC GSH during energy restriction suggests that the change is likely a physiological adaptation to enhance immunocapcity to face the challenges of a state of energy deficit. It is possible that during energy restriction GSH in erythrocytes and other tissues is transported to WBC and/or synthesis of GSH in other tissues is slowed in deference to higher priority WBC GSH synthesis, making total energy intake, rather than specific protein or amino acid intake of greatest importance to GSH synthesis. We observed a 5% decrease in GSH in whole blood during energy restriction, although it

was not significant. While some have associated WBC GSH increases with improved performance (37), the possibility exists that only bolstering concentrations of GSH in erythrocytes or other tissues besides WBC, such as muscle, produces ergogenic results and that these were not observed in our study due to the stress response elicited by energy restriction.

Sen et al. (36) reported a 50% reduction in time-to-exhaustion in rats treated with BSO, a chemical that inhibits GSH synthesis. Similarly, Kramer and colleagues (49) observed a reduction in swim performance of rats given diethyl maleate, another GSH synthesis inhibitor. These studies suggest that GSH has a role in the delay of fatigue during aerobic exercise, but not that enhancing GSH stores is ergogenic, only that the interruption of GSH synthesis may be detrimental. Although Reid and colleagues (49) successfully retarded fatigue in electrically stimulated tibialis anterior muscles with the cysteine-donor NAC, not all data have shown this compound to be ergogenic. A recent study (41) infused the NAC intravenously into non-trained individuals performing intermittent exercise. While NAC infusion was found to increase levels of GSH in the blood, this did not affect performance. Another study (42) did not show NAC to improve performance on a cycling protocol very similar to the one employed in this study. The ability of GSH to delay fatigue and improve athletic performance remains questionable. Our study does not support the efficacy of a whey protein supplement to increase blood GSH or performance in healthy, well-trained individuals.

There has been substantial research on the effect of various energy restriction protocols on aerobic and anaerobic exercise performance. Many investigators have observed decreases in athletic performance during and/or following caloric deficit (59, 60, 62). Others, however, have not seen negative effects (65). In our study, most subjects exhibited decreased time-to-exhaustion following energy restriction, but this observation was not unanimous, with several subjects achieving top performance on the test completing the energy restriction period. Our inability to control certain variables related to performance may have led to reduced reliability in our performance test. As the cyclists were in the middle of their competitive season during the experimental period, most were unwilling to abstain from training and/or competition in the days prior to an exercise test. As a result, the level of fatigue and depletion of glycogen stores a

subject may have suffered prior to each exercise test may have varied between tests for the same subject, possibly impacting performance (2). In addition, although subjects were familiarized with the cycle ergometer used in the submaximal exercise tests at the time of VO_{2peak} testing, they were not familiarized with the submaximal exercise and time-to-exhaustion test protocols prior to their first test on d 1. As a result, a learning effect cannot be discounted from having had an impact on time-to-exhaustion measures.

While the role of circulating AA in the onset of fatigue is an active area of investigation (7), any relationship is currently far from conclusive. Lehmann and colleagues (38) did not observe a correlation between pre- or post-race concentrations of plasma AA and performance in subjects completing an extended endurance competition. Correlations between AA levels and time-to-exhaustion performance were not observed in our subjects either.

Plasma Amino Acids

Citing data from several studies on wasting diseases (20, 27, 66), Droge and Holm (19) have hypothesized that concentrations of plasma free cysteine and glutamine may regulate body protein catabolism and in turn predict loss of lean tissue in conditions involving a cytokine response, increased insulin levels or responsiveness, or increased Cori cycle activity. Kinscherf et al. (35) observed such an association in healthy individuals participating in an exercise program. In addition, these researchers reported that supplementation with 400 mg $3x \cdot wk^{-1}$ exogenous cysteine via NAC curtailed BCM loss predicted by low plasma cysteine levels. The critical role for glutamine in anabolic/catabolic balance has been known for some time, with various methods being attempted to boost stores of the AA. As direct supplementation with glutamine is problematic, due to its oxidation within the intestinal tract and absorption by other organs, it has been hypothesized that exogenous cysteine may help abate the loss of lean tissue in individuals at risk for this occurrence.

Whey protein is relatively high in cysteine, containing 7.5 times more than the casein used in this study (Table 1). We did not perform measures of plasma AA concentrations before beginning the protein supplement intervention with our subjects, however, on d 14, following 13 d of supplementation with 40 $g \cdot d^{-1}$ of casein or whey,

there was no difference between groups in postabsorptive levels of plasma cysteine. Additionally, there were no differences between groups at any time point in the absolute concentration or change in concentration of cysteine. A positive correlation between cumulative NBAL and the change in postabsorptive cysteine levels from d 14 to d 21 was seen in CAS. This observation supports the conclusions of Droge and Holm (19) and Kinscherf et al. (35) – that increasing plasma cysteine concentrations in those at risk for lean tissue loss may reduce the degree of the negative occurrence. The correlation was unexpected from CAS, however, in that WHEY consumed a much larger amount of cysteine throughout the experimental period and was thus thought to be more likely to experience improved levels of free plasma cysteine. It is possible that the absorption and utilization of the casein administered in this experiment favors the appearance of free cysteine in the plasma more than does the whey, helping to boost the plasma cysteine levels in those most at risk for loss of body protein in CAS.

Kashyap et al. (34) compared plasma AA in premature infants fed an 18% whey formula or 60% whey formula, the latter having twice the cysteine content of the former. While differences between treatments did not reach significance, mean plasma cysteine concentrations were approximately 20% higher in subjects receiving the whey-predominant formula. Gaull et al. (24) reported significantly higher plasma cysteine in infants consuming a whey-predominant formula relative to those consuming breast milk. Despite these findings of increased plasma cysteine after whey protein consumption in infants, similar effects have yet to be seen in healthy adults. Additionally, results of a study by Bounous and Kongshavn (10) showed no difference in plasma cysteine levels in rats receiving lactalbumin, a major component of whey protein which is responsible for a significant portion of its cysteine content, or casein for 2 wk, despite the fact that the lactalbumin had five times the cysteine content of the casein administered.

Owing to the identical AA content of the formula diet vehicle consumed with the protein supplements 2 h prior to postprandial measurements in our study, actual AA content of the two meals were quite similar. Despite this, there were significant interactions witnessed between group and state for several AA. Interestingly, these interactions involved histidine, phenlyalanine and tryptophan, AA found in relatively equal concentrations in both the meals given to WHEY and CAS. This punctuates the

variability of absorption, transport and peripheral appearance of AA derived from varying protein and peptide sources.

Our analyses of plasma AA detected only free AA, not peptides or AA bound to proteins. Cysteine bound to any other AA in a peptide, such as glutamate in glutamylcysteine, or in the tripeptide GSH, would not have been detected. Due to differential absorption, clearance and catabolism of dietary proteins, peptides and AA, the plasma free AA profile may differ greatly from that of the ingested protein (57). While our WHEY subjects did not exhibit increased plasma cysteine levels, the possibility that other tissues may have experienced increased cysteine concentrations cannot be discounted. Stipanuk et al. (57) observed increasing hepatic cysteine concentrations with increasing protein intake in rats. Supplementary methionine, but not cysteine, was also associated with greater liver cysteine levels. Additionally, significant amounts of dietary AA are metabolized within the small intestine, resulting in far less than 100% of AA consumed actually being present in peripheral circulation (64). Furthermore, as we did not measure baseline levels of plasma AA, the possibility exists that WHEY may have exhibited lower plasma cysteine levels than CAS at baseline, but these levels were brought to equal those of CAS following 2 wk of supplementation with whey protein. Finally, individual AA may experience concentration maxima at different times postprandially. Although Hall et al. (28) observed peak total plasma AA concentrations throughout a period between 45 and 180 min after meal consumption in subjects given test meals containing whey or casein, the timing of peak concentrations of individual AA were not presented and it is unclear if cysteine was measured. It is possible that by taking postprandial AA samples 2 h following meal ingestion that the cysteine concentrations detected were not peak postprandial concentrations or that concentrations peaked at different times in the two groups, as whey and casein are digested at different rates (23). This appears unlikely however, as Bergstrom et al. (6) fed subjects a meal high in serum albumin and observed peak changes in all plasma AA that significantly differed in concentration from baseline (except methionine and glutamate) at both 1 and 3 h postprandially.

A characteristic of Droge and Holm's hypothesis connecting plasma cysteine and glutamine levels to the loss of lean tissue is the supposition that, during times of stress

and/or increased urea production, skeletal muscle is proteolyzed in order to release free cysteine and glutamine to maintain or increase levels of these two AA in the plasma. Kinscherf et al. (35) observed correlations between these two AA as well as arginine with loss of BCM in healthy individuals. They also observed correlations between percent change in cystine, glutamine, glycine and tyrosine and percent change in BCM over the experimental period. Additionally, Holm et al. (30) claim increases in postabsorptive concentrations in glutamate to be characteristic of catabolism, however, in our study postabsorptive levels of glutamate decreased, although not significantly, in the energy restriction phase. No correlations with NBAL were found in any of the above AA in our study, suggesting that perhaps these correlations do not hold for well-trained athletes. It is worthy of note, however, that our subject numbers were much smaller than those in the above studies, therefore increasing the difficulty of finding moderately-strong correlations.

Correlations between BCAA concentration and cumulative NBAL were detected at 3 of the 4 time points for measurement of plasma AA in WHEY. Branched chain AA can be used as a fuel source for skeletal muscle during exercise. It is possible that high circulating levels of BCAA may inhibit proteolysis for the release of BCAA for use as energy in calorically restricted individuals. Branched chain AA are also suggested to stimulate protein synthesis and/or retard proteolysis. The BCAA leucine is believed to function both as a substrate and signal in protein synthesis (3). Supplementation with BCAA has been associated with more positive NBAL than supplementation with other AA in healthy subjects undergoing bed rest (56). Additionally, Fisler et al. (21) reported correlations between baseline BCAA levels and NBAL in dieting obese persons. The whey used in our study had approximately 15% more BCAA than did the casein. Although there was no effect of group on levels of BCAA in our study, Hall et al. (28) observed significantly greater increases in postprandial plasma free valine, isoleucine and leucine in subjects consuming a meal high in whey content versus those subjects receiving a casein meal. Further, other researchers (45) observed that supplementing with BCAA prevented decreases in plasma glutamine elicited by long-term exercise. This implicates BCAA in a proteolysis-regulating role similar to that proposed for cysteine – as a substrate used to regulate plasma free glutamine concentrations – by

Droge and Holm (19). The correlations observed in our study suggest that levels of plasma BCAA may influence loss of nitrogen during energy restriction in trained athletes. The segregation of the correlation to only those subjects consuming whey, however, hints that levels of these AA may only have importance in a limited range of physiological situations.

Summary

The results of this study show that short-term energy restriction in well-trained aerobic athletes with prior and continued supplementation with casein or whey protein isolate supplements resulted in negative NBAL, increased WBC GSH and tended to negatively impact performance on a timed test to exhaustion. There were not differing effects by protein type on weight loss, NBAL, GSH or performance. While specific protein affected levels of several AA, feeding state and energy restriction exerted wider influence over plasma free AA concentrations. Levels of several AA in the plasma, including BCAA in WHEY and cysteine in CAS, were found to correlate with cumulative NBAL. Our data do not support the ability of a specially-prepared whey protein isolate to affect stores of GSH in whole blood and WBC differently than casein over 3 wk of supplementation. This whey protein isolate also does not appear to affect body nitrogen loss via influence on plasma free cysteine levels. Although our data does not support superiority of either casein or whey isolate on NBAL or performance in dieting athletes, it suggests that circulating levels of certain free AA may influence loss of body nitrogen. Thus, further research on the mechanisms behind the possible influences of plasma AA on NBAL and dietary supplements with which to best manipulate plasma concentrations of AA in trained athletes is warranted.

References

1. Abumrad, N. N. and B. Miller. The physiologic and nutritional significance of plasma-free amino acid levels. *JPEN.* 7:163-170, 1983.
2. Ahlborg, B., J. Bergstrom, G. Edelund, and E. Hultman. Muscle glycogen and muscle electrolytes during prolonged physical exercise. *Acta Physiol. Scand.* 70:129-142, 1967.
3. Anthony, J. C., T. G. Anthony, S. R. Kimball, and L. S. Jefferson. Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. *J. Nutr.* 131:856S-860S, 2001.
4. Antonio, J, M. S. Sanders, and D. Van Gammeren. The effects of bovine colostrum supplementation on body composition and exercise performance in active men and women. *Nutrition.* 17: 243-247, 2001.
5. Barclay, J. K. and M. Hansel. Free radicals may contribute to oxidative skeletal muscle fatigue. *Can. J. Physiol. Pharmacol*. 69:279-284, 1991.
6. Bergstrom, J., P. Furst, and E. Vinnars. Effect of a test meal, without and with protein, on muscle and plasma free amino acids. *Clin. Sci.* 79:331-337, 1990.
7. Blomstrand, E. Amino acids and central fatigue. *Amino Acids.* 20:25-34, 2001.
8. Boirie, Y., M. Dangin, P. Gachon, M-P. Vasson, J-L. Maubois, and B. Beaufrere. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc. Natl. Acad. Sci. USA*. 94:14930-14935, 1997.
9. Bounous, G. Whey protein concentrate and glutathione modulation in cancer treatment. *Anticancer Res*. 20:4785-4792, 2000.
10. Bounous, G. and P. A. L. Konghsvn. Influence of protein type in nutritionally adequate diets on the development of immunity. In Absorption and Utilization of Amino Acids, Volume II. ed. M. Friedman, CRC Press, Boca Raton FL, pp 219-233, 1989.
11. Bridgeman, M. M., M. Marsden, W. MacNee, D. C. Flenley, and A. P. Ryle. Cysteine and glutathione concentrations in plasma and bronchoalveolar lavage fluid after treatment with N-acetylcysteine. *Thorax.* 46:39-42, 1991.

12. Brinkworth, G. D., J. D. Buckley, P. C. Bourndon, J. P. Gulbin, and A. David. Oral bovine colostrum supplementation enhances buffer capacity but not rowing performance in elite female rowers. *Int. J. Sport Nutr. Exerc. Metab.* 12:349-365, 2002.
13. Buckley, J. D., M. J. Abbott, G. D. Brinkworth, and P. B. Whyte. Bovine colostrum supplementation during endurance running training improves recovery, but not performance. *J. Sci. Med. Sport.* 5:65-79, 2002.
14. Burke, D. G., P. D. Chilibeck, K. S. Davidson, D. G. Candow, J. Farthing, and T. Smith-Palmer. The effect of whey protein supplementation with and without creatine monohydrate combined with resistance training on lean tissue mass and muscle strength. *Int. J. Sport Nutr. Exerc. Metab.* 11:349-364, 2001.
15. Coombes, J. S., M. Conacher, S. K. Austen, and P. A. Marshall. Dose effects of oral bovine colostrum on physical work capacity in cyclists. *Med. Sci. Sports Exerc.* 34:1184-1188, 2002.
16. Dangin, M., Y. Boirie, C. Guillet, and B. Beaufrere. Influence of the protein digestion rate on protein turnover in young and elderly subjects. *J. Nutr.* 132:3228S-3233S, 2002.
17. Davies, K. J., A. T. Quintanilha, G. A. Brooks, and L. Packer. Free radicals and tissue damage produced by exercise. *Biochem. Biophys. Res. Commun.* 107:1198-1205, 1982.
18. Demling, R. H. and L. DeSanti. Effect of a hypocaloric diet, increased protein intake and resistance training on lean mass gains and fat mass loss in overweight police officers. *Ann. Nutr. Metab.* 44:21-29, 2000.
19. Droge, W. and E. Holm. Role of cysteine and glutathione in HIV infection and other diseases associated with muscle wasting and immunological dysfunction. *FASEB J.* 11:1077-1089, 1997.
20. Eriksson, L. S. Splanchnic exchange of glucose, amino acids and free fatty acids in patients with chronic inflammatory bowel disease. *Gut.* 24:1161-1168, 1983.
21. Fisler, J. S., E. M. Kaptein, E. J. Drenick, J. T. Nicoloff, N. N. Yoshimura, and M. E. Swenseid. Metabolic and hormonal factors as predictors of nitrogen retention in obese men consuming very low calorie diets. *Metabolism.* 34:101-105, 1985.

22. Fogelholm, M. and H. Hiilloskorpi. Weight and diet concerns in Finnish female and male athletes. *Med. Sci. Sports. Exer.* 31:229-235, 1999.
23. Fruhbeck, G. Slow and fast dietary proteins. *Nature.* 391:843-4, 1998.
24. Gaull, G. E., D. K. Rassin, N. C. Raiha, and K. Heionen. Milk protein quantity and quality in low-birth-weight infants. III. Effects on sulfur amino acids in plasma and urine. *J. Pediatr.* 90:348-355, 1977.
25. Greenwood, M., J. Farris, R. Kreider, L. Greenwood, and A. Byars. Creatine supplementation patterns and perceived effects in select division I collegiate athletes. *Clin. J. Sport. Med.* 10:191-194, 2000.
26. Ha, E. and M. B. Zemel. Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people. *J. Nutr. Biochem.* 14:251-258, 2003.
27. Hack, V., D. Schmid, R. Breitkreutz, C. Stahl-Henning, P. Drings, R. Kinscherf, F. Taut, E. Holm, and W. Droge. Cystine levels, cystine flux, and protein catabolism in cancer cachexia, HIV/SIV infection, and senescence. *FASEB J.* 11:84-92, 1997.
28. Hall, W. L., D. J. Millward, S. J. Long, and L. M. Morgan. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *Br. J. Nutr.* 89: 239-248, 2003.
29. Hofman, Z., R. Smeets, G. Verlaan, R. Lugt, P.A. Verstappen. The effect of bovine colostrum supplementation on exercise performance in elite field hockey players. *Int. J. Sport Nutr. Exerc. Metab.* 12:461-469, 2002.
30. Holm, E., V. Hack, M. Tokus, R. Breitkreutz, A. Babylon, and W. Droge. Linkage between postabsorptive amino acid release and glutamate uptake in skeletal muscle tissue of healthy young subjects, cancer patients, and the elderly. *J. Mol. Med.* 75:454-461, 1997.
31. Institute of Medicine of the National Academies. *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids.* Washingon, DC: The National Academies Press, pp. 465-608.
32. Jackson, A. S. and M. L. Pollock. Generalized equations for predicting body density in men. *Br. J. Nutr.* 40:497-504, 1978.

33. Jenkins, R. R. Symposium: oxidant stress, aging, and exercise. *Med. Sci. Sports Exerc.* 25:210-236, 1993.
34. Kashyap, S., E. Okamoto, S. Kanaya, C. Zucker, K. Abildskov, R. B. Dell, and W. C. Heird. Protein quality in feeding low birth weight infants: a comparison of whey-predominant versus casein-predominant formulas. *Pediatrics.* 79:748-755, 1987.
35. Kinscherf, R., V. Hack, T. Fischbach, B. Freidmann, C. Weiss, L. Edler, P. Bartsch, and W. Droge. Low plasma glutamine in combination with high glutamate levels indicate risk for loss of body cell mass in healthy individuals: the effect of N-acetyl-cysteine. *J. Mol. Med*. 74:393-400, 1996.
36. Kramer, K., H. Dijkstra, A. Bast. Control of physical exercise of rats in a swimming basin. *Physiol. Behav.* 53:271-276, 1993.
37. Lands, L. C., V. L. Grey, A. A. Smountas. Effect of supplementation with a cysteine donor on muscular performance. *J. Appl. Physiol*. 87:1381-1385, 1999.
38. Lehmann, M., M. Huonker, F. Dimeo, N. Heinz, U. Gastmann, N. Treis, J. M. Steinaccker, J. Keul, R. Kajewski, and D. Haussinger. Serum amino acid concentrations in nine athletes before the after the 1993 Colmar ultra triathlon. *Int. J. Sports Med.* 16:155-159, 1995.
39. Mahe, S., N. Roos, R. Benamouzig, L. Davin, C. Luengo, L. Gagnon, N. Gausserges, J. Rautureau, and D. Tome. Gastrojejunal kinetics and the digestion of [15N] beta-lactoglobulin and casein in humans: the influence of the nature and quantity of the protein. *Am. J. Clin. Nutr.* 63:546-553, 1996.
40. McArdle, A., D. Pattwell, A. Vasilaki, R. D. Griffiths, and M. J. Jackson. Contractile activity-induced oxidative stress: cellular origin and adaptive responses. *Am. J. Physiol. Cell. Physiol*. 280:C621-627, 2001.
41. Medved, I., M. J. Brown, A. R. Bjorksten, J. A. Leppik, S. Sostaric, and M. J. McKenna. N-acetylcysteine infusion alters blood redox status but not time to fatigue during intense exercise in humans. *J. Appl. Physiol.* 94:1572-1582, 2003.
42. Medved, I., M. J. Brown, A. R. Bjorksten, and M. J. McKenna. Effects of intravenous N-acetylcysteine infusion on time to fatigue and potassium regulation during prolonged exercise. *J. Appl. Physiol.* 96:211-217, 2004.

43. Mourier, A., A. X. Bigard, E. de Kerviler, B. Roger, H. Legrand, C. Y. Guezennec. Combined effects of caloric restriction and branched-chain amino acid supplementation on body composition and exercise performance in elite wrestlers. *Int. J. Sports Med.* 18:47-55, 1997.
44. Opplinger, R. A., S. A. Steen, and J. R. Scott. Weight loss practices of college wrestlers. *Int. J. Sport. Nutr. Exerc. Metab.* 13:29-46, 2003.
45. Parry-Billings, M., R. Budgett, Y. Koutedakis, E. Blomstrand, S. Brooks, C. Williams, P. C. Calder, S. Pilling, R. Baigrie, and E. Newsholme. Plasma amino acid concentration in the overtraining syndrome: Possible effects on the immune system. *Med. Sci. Sports Exerc.* 24:1353-1358, 1992.
46. Piatti, P. M., L. D. Monti, F. Magni, I. Fermo, L. Baruffaldi, R. Nasser, G. Santambrogio, M. C. Librenti, M. Galli-Kienle, A. E. Pontiroli, and G. Pozza. Hypocaloric high-protein diet improves glucose oxidation and spares lean body mass: comparison to hypocaloric high-carbohydrate diet. *Metabolism.* 43:1481-1487, 1994.
47. Poullain, M. G., J. P. Cezard, L. Roger, and F. Mendy. Effect of whey proteins, their oligopeptide hydrolysates and free amino acid mixtures on growth and nitrogen retention in fed and starved rats. *JPEN.* 13:382-6, 1989.
48. Rand, W. M., P. L. Pellett, and V. R. Young. Meta-analysis of nitrogen balance studies for estimating protein requirements in healthy adults. *Am. J. Clin. Nutr.* 77:109-127, 2003.
49. Reid, M. B., D. S. Stokic, S. M. Koch, F. A. Khawli, and A. A. Leis. N-Acetylcysteine inhibits muscle fatigue in humans. *J. Clin. Invest.* 94:2468-2474, 1994.
50. Robertson, J., R. Maughan, G. Duthie and P. Morrice. Increased blood antioxidant systems of runners in response to training. *Clin. Sci*. 80:611-618, 1991.
51. Rush, J. W. and S. D. Sandiford. Plasma glutathione peroxidase in healthy young adults: influence of gender and physical activity. *Clin. Biochem.* 36:345-351, 2003.

52. Sen, C. K., M. Atalay, O. Hanninen. Exercise-induced oxidative stress: glutathione supplementation and deficiency. *J. Appl. Physiol*. 77:2177-2187, 1994.
53. Smith, A. T., D. R. Clemmons, L. E. Underwood, V. Ben-Ezra, and R. McMurray. The effect of exercise on plasma somatomedin-C/insulinlike growth factor I concentrations. *Metabolism*. 36:533-537, 1987.
54. Smith, W. J., L. E. Underwood, and D. R. Clemmons. Effects of caloric or protein restriction on insulin-like growth factor-I (IGF-I) and IGF-binding proteins in children and adults. *J. Clin. Endocrinol. Metab.* 80:443-449, 1995.
55. Stanko, R. T., D. L. Tietze, and J. E. Arch. Body composition, nitrogen metabolism, and energy utilization with feeding of mildly restricted (4.2 MJ/d) and severely resticted (2.1 MJ/d) isonitrogenous diets. *Am. J. Clin. Nutr.* 56:636-640, 1992.
56. Stein, T. P., M. D. Schluter, M. J. Leskiw, and G. Boden. Attenuation of the protein wasting associated with bed rest by branched-chain amino acids. *Nutrition*. 15:656-660, 1999.
57. Stipanuk, M. H., M. Londono, J. I. Lee, M. Hu, A. F. Yu. Enzymes and metabolites of cysteine metabolism in nonhepatic tissues of rats show little response to changes in dietary protein or sulfur amino acids levels. *J. Nutr.* 132:3369-3378, 2002.
58. Van Loan, M. D., N. L. Keim, T. F. Barbieri, and P. L. Mayclin. The effects of endurance exercise with and without a reduction of energy intake on fat-free mass and the composition of fat-free mass in obese women. *Eur. J. Clin. Nutr.* 48:408-415, 1994.
59. Velthuis-te Wierik, E. J. M., L. V. Hoogzaad, H. van den Berg, and G. Schaafsma. Effects of moderate energy restriction on physical performance and substrate utilization in non-obese men. *Int. J. Sports Med.* 15:478-484, 1994.
60. Walberg, J. L., V. K. Ruiz, S. L. Tarlton, D. E. Hinkle, F. W. Thye. Exercise capacity and nitrogen loss during a high or low carbohydrate diet. *Med. Sci. Sports Exer.* 20:34-43, 1988.

61. Walberg-Rankin, J. Changing body weight and composition in athletes. In Perspectives in Exercise and Sports Medicine vol 11 : Exercise, Nutrition, and Control of Body Weight. eds. D. Lamb and R. Murray, Cooper Publ., Carmel IN, pp 199-242, 1998.
62. Webster, S., R. Rutt, A. Welstman. Physiological effects of a weight loss regimen practiced by college wrestlers. *Med. Sci. Sports Exerc.* 22:229-234, 1990.
63. Wilmore, J. H. Body composition in sport and exercise: directions for future research. *Med. Sci. Sports Exer.* 15:21-31, 1983.
64. Wu, G. Intestinal mucosal amino acid catabolism. *J. Nutr.* 128:1249-1252, 1998.
65. Zachwieja, J. J., D. M. Ezell, A. D. Cline, J. C. Ricketts, P. C. Vicknair, S. M. Schorle, and D. H. Ryan. Short-term dietary energy restriction reduces lean body mass but not performance in physically active men and women. *Int. J. Sports Med.* 22:310-316, 2001.
66. Zhang, P. C. and C. P. Pang. Plasma amino acid patterns in cancer. *Clin. Chem.* 38:1198-1199, 1992.

Table 1. Amino Acid content of protein supplements consumed by WHEY and CAS, and Ensure® formula diet.

	AA content (g/kg)		Total AA in acute dose (g)						
	Whey	**Casein**	**Whey**	**OR**	**Casein**	**+**	**Ensure®**	**=**	**WHEY**	**CAS**
Ala	50	2.8	1.00		0.06		0.31		1.31	0.36
Arg	15	34	0.30		0.68		0.35		0.65	1.03
Asp	114	63	2.28		1.26		0.74		3.02	2.00
Cys	23	3.0	0.46		0.06		0.08		0.54	0.14
Glu	177	205	3.54		4.10		1.78		5.32	5.88
Gly	18	16	0.36		0.32		0.20		0.56	0.52
His	16	25	0.32		0.50		0.22		0.54	0.72
Ile	61	47	1.22		0.94		0.43		1.65	1.37
Leu	98	82	1.96		1.64		0.79		2.75	2.43
Lys	86	72	1.72		1.44		0.64		2.36	2.08
Met	23	19	0.46		0.38		0.21		0.67	0.59
Phe	28	44	0.56		0.88		0.41		0.97	1.29
Pro	57	95	1.14		1.90		0.77		1.91	2.67
Ser	48	50	0.96		1.00		0.48		1.44	1.48
Thr	75	38	1.50		0.76		0.38		1.88	1.14
Trp	18	16	0.36		0.32		0.11		0.47	0.43
Tyr	28	47	0.56		0.94		0.39		0.95	1.33
Val	58	60	1.16		1.20		0.50		1.66	1.70

Note: The numbers on the far right indicate total amino acid content, in grams, of the meal consumed 2 h prior to postprandial plasma AA measures on d 14 and d 21(1 can Ensure® – 237mL/250kcal + 20 g protein supplement).

Sources: AmmunoMed Llc., Toledo, OH; ICN Biomedicals, Aurora, OH; Abbott Laboratories, Abbott Park, IL

Table 2. Baseline subject characteristics

	WHEY (n=10)	**CAS (n=10)**
Age (yrs)	21.7 ± 0.7	23.4 ± 1.0
VO_{2peak} (mL $0_2 \cdot kg^{-1} \cdot min^{-1}$)	60.7 ± 2.2	56.3 ± 2.3
BF %	10.0 ± 1.3	11.4 ± 1.1
Weight (kg)	71.9 ± 1.8	73.0 ± 1.6
Training Volume ($min \cdot wk^{-1}$)	304 ± 30	381 ± 37

Note: All values mean ± SE

Table 3. Postabsorptive (PA) and postprandial (PP) plasma free AA concentrations on d 14 (energy balance) and d 21 (during energy restriction).

	WHEY	CAS	WHEY	CAS	WHEY	CAS	WHEY	CAS
AA (μM)	Day 14 - PA	Day 14 - PA	Day 14 - PP	Day 14 - PP	Day 21 - PA	Day 21 - PA	Day 21 - PP	Day 21 – PP
Ala	486 ± 27.9	490 ± 31.2	616 ± 20.4	603 ± 31.8	467 ± 13.4	464 ± 32.5	628 ± 28.7	613 ± 47.7
Aab	29.0 ± 2.0	30.0 ± 1.5	29.5 ± 3.0	31.1 ± 2.5	48.8 ± 3.3	41.6 ± 3.5	42.9 ± 2.9	42.6 ± 4.3
Arg	83.0 ± 6.7	89.4 ± 5.1	90.9 ± 6.2	98.7 ± 5.8	81.8 ± 5.3	89.6 ± 3.7	79.1 ± 4.8	96.7 ± 3.8
Asn	91.2 ± 5.6	99.0 ± 5.7	108 ± 6.8	113 ± 4.6	98.1 ± 3.6	99.8 ± 5.6	103 ± 5.8	116 ± 6.6
Cit	45.8 ± 1.8	45.4 ± 2.4	48.9 ± 2.1	49.3 ± 2.7	48.1 ± 1.4	50.3 ± 2.1	48.5 ± 2.4	52.3 ± 2.6
Cys	42.2 ± 3.8	46.6 ± 4.2	46.4 ± 4.5	47.0 ± 4.7	47.9 ± 5.7	49.2 ± 5.1	51.6 ± 6.4	55.0 ± 7.7
Glu	29.0 ± 3.5	26.8 ± 3.5	31.9 ± 3.3	29.3 ± 3.0	25.0 ± 1.9	20.8 ± 1.8	27.3 ± 2.9	27.6 ± 3.6
Gln	697 ± 28.5	682 ± 9.7	692 ± 20.3	703 ± 16.5	704 ± 21.8	664 ± 14.3	658 ± 19.7	654 ± 9.4
Gly	298 ± 16.5	314 ± 20.2	281 ± 15.7	306 ± 20.0	303 ± 10.7	301 ± 21.1	272 ± 19.8	295 ± 22.4
His	80.9 ± 3.2	88.5 ± 3.0	80.9 ± 3.5	94.7 ± 2.3	89.6 ± 2.4	89.0 ± 4.9	81.7 ± 3.1	93.7 ± 2.1
Ile	89.9 ± 5.2	99.6 ± 5.2	143 ± 12.4	131 ± 7.3	91.2 ± 5.3	98.7 ± 5.0	134 ± 11.8	129 ± 7.6
Leu	192 ± 8.9	199 ± 6.5	274 ± 20.6	253 ± 12.8	206 ± 7.0	222 ± 11.0	265 ± 20.2	261 ± 18.2
Lys	157 ± 7.3	168 ± 8.0	224 ± 10.4	217 ± 11.3	164 ± 7.9	175 ± 8.5	199 ± 12.1	222 ± 11.5
Met	34.3 ± 1.5	38.5 ± 2.5	40.6 ± 2.1	48.2 ± 3.3	39.1 ± 1.3	40.6 ± 2.5	40.4 ± 2.3	49.1 ± 3.6
Orn	32.4 ± 2.5	38.8 ± 3.8	40.7 ± 2.3	48.74 ± 3.7	29.3 ± 2.3	31.3 ± 1.8	34.0 ± 2.2	41.5 ± 2.8
Phe	65.0 ± 1.7	71.1 ± 2.4	65.1 ± 1.6	78.1 ± 2.2	71.8 ± 3.0	76.0 ± 2.1	68.1 ± 2.9	82.1 ± 2.8
Pro	294 ± 34.5	365 ± 48.3	377 ± 32.6	464 ± 48.7	216 ± 13.5	280 ± 45.8	298 ± 20.0	408 ± 37.0
Ser	88.8 ± 3.8	104 ± 4.9	101 ± 5.8	119 ± 4.0	104 ± 4.2	109 ± 4.3	101 ± 6.9	122 ± 7.3
Tau	62.4 ± 6.0	65.0 ± 4.9	60.0 ± 6.9	63.2 ± 4.8	63.8 ± 5.8	60.9 ± 4.3	58.9 ± 6.6	54.8 ± 3.7
Thr	191 ± 9.4	182 ± 10.4	228 ± 9.6	200 ± 10.5	191 ± 5.7	182 ± 13.0	207.1 ± 10.4	195 ± 14.6
Trp	69.1 ± 2.5	72.8 ± 4.9	103 ± 5.8	83.7 ± 6.4	65.1 ± 2.6	69.2 ± 4.6	101 ± 6.0	90.3 ± 6.2
Tyr	70.1 ± 7.5	90.6 ± 6.0	100 ± 5.5	121 ± 6.9	77.7 ± 2.6	88.2 ± 6.1	94.3 ± 4.5	115 ± 7.5
Val	342 ± 20.5	367 ± 16.6	436 ± 25.7	458 ± 20.8	343 ± 12.0	375 ± 15.0	423 ± 23.8	465 ± 24.6
BCAA	620 ± 33.6	666 ± 26.7	852 ± 57.3	841 ± 39.7	640 ± 23.9	695 ± 25.9	821 ± 55.3	855 ± 48.9
EAA	1220 ± 44.1	1290 ± 37.0	1590 ± 74.3	1560 ± 54.3	1260 ± 28.4	1330 ± 31.1	1520 ± 81.8	1590 ± 68.1
NEAA	2210 ± 104	2340 ± 70.5	2490 ± 81.6	2630 ± 86.7	2160 ± 37.9	2200 ± 102	2350 ± 89.0	2540 ± 122

Significant main effects of group, state and time are displayed in Table 4.
Note: All values mean ± SE (μM)

Table 4. Main effects of group, day and state on plasma free AA concentration

	Main Effect of Group	
AA	**CAS**	**WHEY**
Tyr	103.9 ± 4.2	85.5 ± 4.2

	Main Effect of Day	
AA	**Day 14**	**Day 21**
Ala	476.6 ± 13.3	615.1 ± 16.2
Cit	47.3 ± 1.4	49.8 ± 1.4
Cys	45.5 ± 2.9	50.9 ± 4.3
Glu	29.2 ± 2.2	25.2 ± 1.7
Orn	40.2 ± 2.2	34.0 ± 1.5
Pro	375.1 ± 27.9	300.4 ± 20.6
Sulfur (Met + Cys)	85.9 ± 3.4	93.2 ± 5.0

	Main Effect of State	
AA	**Postabsorptive**	**Postprandial**
Cys	46.5 ± 3.2	50.0 ± 4.1
Glu	25.4 ± 1.8	29.0 ± 2.2
Gly	303.9 ± 11.6	288.4 ± 13.2
Ile	94.1 ± 3.2	134.0 ± 6.5
Leu	204.7 ± 4.7	263.3 ± 12.1
Met	38.1 ± 1.3	44.6 ± 1.9
Orn	33.0 ± 1.6	41.2 ± 1.7
Pro	288.5 ± 24.6	386.9 ± 23.1
Tau	63.0 ± 2.5	59.2 ± 2.8
Tyr	81.7 ± 3.4	107.7 ± 3.6
Val	356.7 ± 9.8	445.3 ± 15.4
BCAA	655.4 ± 16.5	842.6 ± 33.3
EAA	1273.5 ± 21.0	1566.2 ± 45.9
NEAA	2228.8 ± 50.9	2500.9 ± 61.7
Sulfur (Met + Cys)	84.6 ± 3.6	94.5 ± 4.9

Note: Means ± SE (μM) of plasma AA exhibiting main effects of group, time or state ($p < 0.05$).

Table 5. Correlations between postabsorbtive (PA) and postprandial (PP) plasma free AA concentrations and changes in concentration during energy restriction with NBAL in WHEY (W), CAS (C) and both groups combined (B)

	Absolute				Change	
AA	**Day 14 – PA**	**Day 14 – PP**	**Day 21 – PA**	**Day 21 – PP**	**PA – Day 14 to Day 21**	**PP – Day 14 to Day 21**
Cit						W (+), B (+)
Cys					C (+)	
His				W (+)		
Ile				W (+)		
Leu	W (+), B (+)	W (+)		W (+)		
Orn	C (−)	C (−)			C (+)	C (+), B (+)
Phe			W (−), B (+)		B (−)	
Pro		C (−)				
Ser				W (+)		W (+)
Trp	C (−)	W (+)		W (+)	C (+)	
Val	W (+)	W (+)			W (−)	
BCAA	W (+)	W (+)		W (+)		

Note: (+) Indicates significant positive correlation ($p \leq 0.05$).
(−) Indicates significant negative correlation ($p \leq 0.05$).

Table 6. White blood cell and whole blood GSH concentrations prior to supplementation (Day 1), after 2 wk supplementation (Day 15), and following 4 d energy restriction with supplementation (Day 22).

		Day	WHEY (n = 10)	CAS (n = 10)
WBC	**GSH**	1	8.4 ± 0.9	8.8 ± 1.0
	(μmol/million cells)	15	9.7 ± 1.1	7.8 ± 1.1
		22	12.4 ± 1.0*	11.5 ± 1.2*
Whole Blood	**GSH**	**Day**	**WHEY (n = 10)**	**CAS (n = 10)**
	(μmol/mg protein)	1	1316 ± 29	1379 ± 35
		15	1446 ± 20	1393 ± 71
		22	1382 ± 33	1313 ± 71

*Indicates significant difference from d 1 and d 15 for both groups combined ($p < 0.01$).
Note: All values mean ± SE

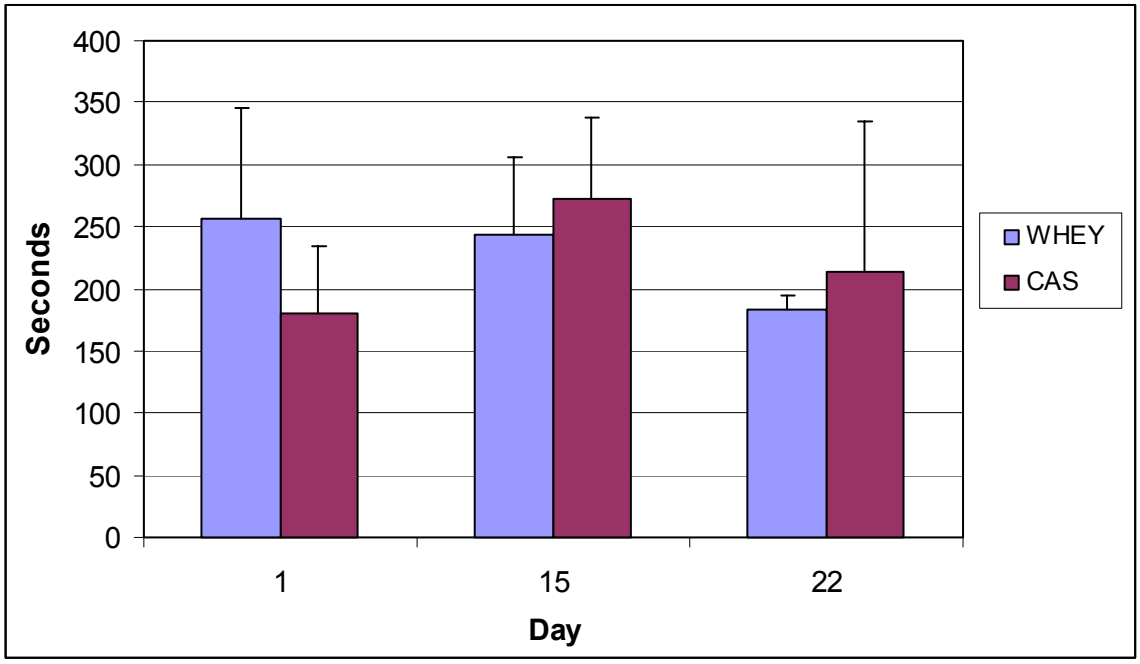

Figure 1. Time to exhaustion at 90% VO_{2peak} following 45 min submaximal exercise, prior to supplementation (Day 1), after 2 wk supplementation (Day 15), and following 4 d energy restriction with supplementation (Day 22).

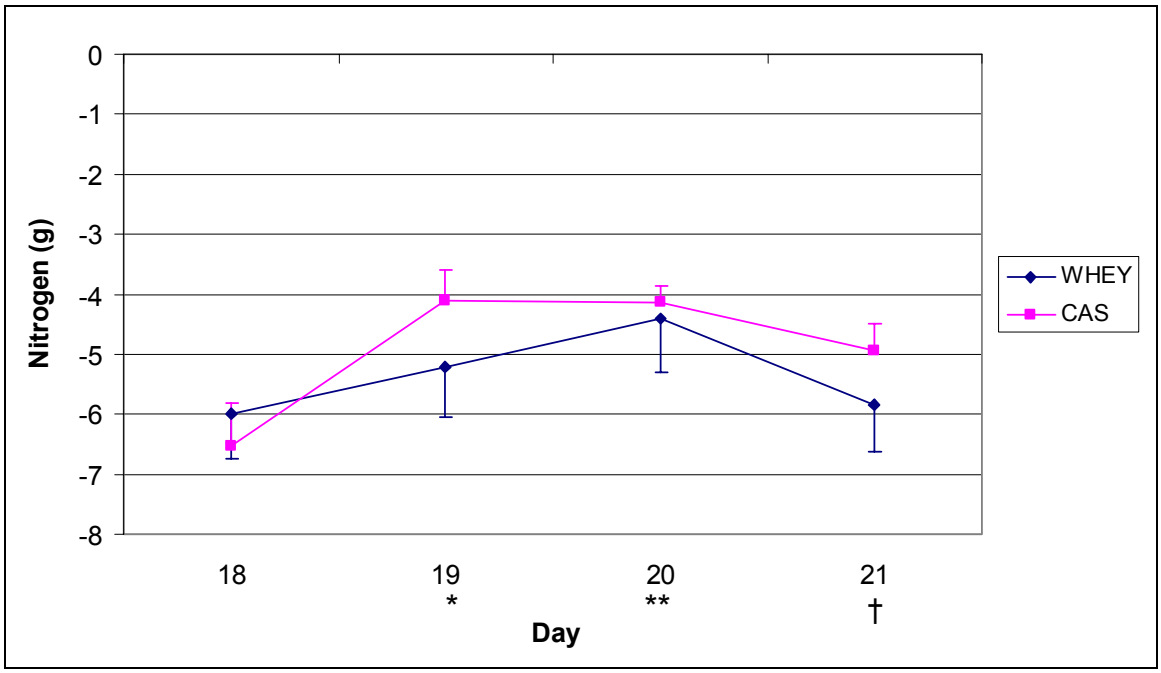

Figure 2. Daily nitrogen balance during energy restriction at 20 kcal·kg^{-1}·d^{-1} plus 40g·d^{-1} protein supplement.
* Indicates significant difference from d 18 for both groups combined (p = 0.013).
** Indicates significant difference from d 18 for both groups combined (p = 0.002).
† Indicates significant difference from d 20 for both groups combined (p = 0.007).

Chapter 4: Summary and Recommendations

Athletes often engage in energy restriction in order to reduce body fat and/or improve athletic performance (35). Many athletes consume nutritional supplements with the same intent. The nutritional supplement industry has become a multi-billion dollar enterprise within the United States and throughout the rest of the world, catering to the desire of athletes and non-athletes alike for a magic bullet with which to control body composition and performance. Unlike food products, nutritional supplements do not undergo FDA scrutiny for safety and efficacy prior to being released on the market. While some nutritional supplements back-up manufacturer claims with health- or performance-related benefits, many fall short of the grandiose claims, some have no effect whatsoever and a number have even been proven harmful. Federal funding is insufficient to test all supplements, so independent laboratories are left to validate the hypothesized benefits and mechanisms of many nutritional supplements.

A large segment of the nutritional supplement industry is devoted to protein supplements. These range from powders to bars to shakes to capsules, containing free amino acids, hydrolyzed proteins, whole proteins or combinations of the three. Whey, a protein available in great supply as a waste byproduct of cheese production, is one of the most popular types of protein supplements on the market today.

Traditionally, whey and most other protein and amino acid supplements were thought of solely as aids for anabolism. Recently, however, other characteristics and the bioactivities of various available protein supplements have been investigated. Protein supplements are now touted to improve memory and brain function, enhance immunity, improve aerobic performance, enhance antioxidant status, control appetite and reduce adipose tissue.

The production of the whey protein isolate supplement investigated in our study, Immunocal®, had several of the above benefits as goals. The processing of the supplement is claimed to avoid the denaturing of glutamylcysteine groups present in components of whey. These dipeptides are then thought to be available for the synthesis of GSH, the most important endogenous antioxidant. By increasing concentrations of GSH, immunity is thought to be enhanced and fatigue, due to exposure to ROS produced during aerobic exercise, delayed (7, 65, 72, 90, 98). Indeed, this product has been shown in several studies to increase plasma (14) and WBC (66) levels of GSH.

In addition to the goal of improved athletic performance, many persons consume nutritional supplements marketed for improved body composition. The changes these people usually desire include either loss of adipose tissue, increased muscle mass, or both. While current recommendations include consumption of amino acids or protein to be beneficial for anabolism in resistance training (105) and that timing of supplementation may be important (33), there is no consensus as to whether a particular protein supplement is able to best prevent the loss of lean tissue in those attempting to lose weight through dieting. Some research has suggested that plasma levels of cysteine and glutamine may affect loss of lean tissue in active persons (62). Protein consumption has been shown to modulate plasma AA levels both acutely and chronically (e.g. 21, 71). Whey protein is particularly rich in the AA cysteine and thus was considered a possible candidate for testing as a supplement with which to avoid lean loss while dieting.

Research has also detected a negative impact on athletic performance during and immediately following an energy-restriction period (108, 112). Science has yet to determine an ideal nutritional supplement for consumption during energy restriction with which to avoid performance losses. The hypothesized prevention of protein loss and supplementation of GSH provided by whey supplementation may help avoid these declines in performance while dieting and was investigated in this study.

The purpose of our study was thus, to determine 1) if whey protein supplementation has a different effect than casein supplementation on loss of body nitrogen during energy restriction, aerobic exercise performance during energy balance and restriction, and postabsorptive and postprandial plasma amino acid concentrations during energy balance and restriction, 2) if initial plasma amino acid concentrations, or their change, are correlated with loss of body nitrogen during energy restriction.

Twenty well-trained, college-aged cyclists were randomly divided into 2 groups that consumed 40g of either whey protein, (WHEY, n = 10) or casein (CAS, n = 10) daily for 3 wk. The last 4 d of the experimental period (d 18 – 21) subjects restricted energy intake to 20 $kcal \cdot kg^{-1} \cdot d^{-1}$ of a liquid formula diet (63.9% CHO, 14.1% PRO, 22% Fat) plus protein supplement. Apparent NBAL was calculated by subtracting estimated dermal and fecal losses and total urinary nitrogen produced during the energy restriction period from dietary nitrogen. On d 14 and 21, postabsorptive and 2 h postprandial (250

kcal formula diet + 20 g protein) AA were assessed. On d 1, 15 and 22 subjects had blood drawn for analysis of whole blood and WBC GSH and performed a cycling bout consisting of 8 min at 70% VO_{2peak} followed by 37 min at 55% VO_{2peak} and a timed test to exhaustion at 90% VO_{2peak} after a standardized breakfast.

Both groups experienced similar daily and cumulative negative NBAL (CAS = -19.7 ± 1.4 g, WHEY = -21.4 ± 2.7 g) during energy restriction. There were trends toward a main effect of day ($p = 0.073$), with reduced performance during energy restriction, and an interaction of day and group ($p = 0.072$), on time-to-exhaustion at 90% VO_{2peak}, as CAS tended toward improved performance on d 15 while WHEY decreased slightly.

There was no significant main effect of group or interaction of group with other factors on plasma cysteine or glutamine, but there was a main effect of group on tyrosine (CAS = 103.9 ± 4.2 μM; WHEY = 85.5 ± 4.2 μM). Interactions between group and state were present for three AA with histidine and phenylalanine with both increasing in the postprandial state in CAS and decreasing in WHEY, while levels of tryptophan increased to a much greater degree in WHEY relative to CAS. Levels of histidine also exhibited a significant interaction between group and day, as concentrations were lower in WHEY than in CAS on d 14 (WHEY = 80.9 ± 2.3 μM; CAS = 91.6 ± 2.0 μM), and concentrations of the AA increased modestly in WHEY during energy restriction while CAS levels remained unchanged. There were also three-way interactions between group, day and state for serine, alpha-aminobutyric acid, lysine and asparagine. WHEY experienced attenuated increases in postprandial lysine, asparagine and serine levels during energy restriction. In addition to greater levels of alpha-aminobutyric acid pre- and post-meal in both groups during energy restriction, WHEY experienced an enhanced postprandial response in levels of the AA while dieting.

Focusing on the original hypotheses, there were significant main effects of state (PA = 46.5 ± 3.2 μM, PP = 50.0 ± 4.1 μM) and day (d 14 = 45.5 ± 2.9 μM, d 21 = 50.9 ± 4.3 μM) on plasma concentrations of cysteine. Additionally, there was a significant interaction of state and day on plasma levels of glutamine with levels increasing 2 h following meal consumption on d 14, but decreasing on d 21.

There were both positive and negative correlations of plasma AA concentrations and changes in the concentrations of multiple AA, including the branched chain AA,

citruline, histidine, ornithine, phenylalanine, proline, serine and tryptophan with NBAL. Of consequence to our original hypothesis, the absolute change in postabsorptive plasma cysteine concentration from d 14 to d 21 was positively correlated with NBAL ($r = 0.766$, $p = 0.01$) in CAS but not in WHEY. There was no correlation between initial levels or changes in plasma glutamine concentration and NBAL in either group.

White blood cell GSH increased 40% ($p = 0.016$) over baseline following energy restriction in both groups combined, but there was no significant difference between treatments. There was a mean increase in whole blood GSH of 9.9% from d 1 (1316 ± 32 μmol/mg protein) to d 15 (1446 ± 52 μmol/mg protein) in WHEY, but it was not significant. There was no change in whole blood GSH in CAS.

Subjects in our study received between 1.09 to 1.30 $g \cdot kg^{-1} \cdot d^{-1}$ PRO (mean = 1.20 $gl \cdot kg^{-1} \cdot d^{-1}$) during the energy restriction period. This is 1.5 times the DRI for protein consumption. Despite these levels of protein, both groups in our study experienced negative daily and cumulative NBAL. The negative NBAL is in agreement with observations in other studies in healthy individuals (100, 102, 110). Although some have suggested that athletes require more than the DRI for protein, official recommendations have not been made, and it is likely that the type and volume of training the athlete performs influences nitrogen needs.

While others have shown casein supplementation to have greater benefits to body composition than whey (30), our data do not suggest a difference as far as body nitrogen is concerned. While it is unlikely that bioactivities of either whey or casein stimulate fat oxidation more than the other, the possibility cannot be discounted, as the proteins have many observed biological activities and other researchers have claimed superiority of one protein or the other for body composition improvement (30, 66).

We are aware of only one previous study of the ergogenicity of the particular whey protein isolate supplement investigated in our study. Lands et al. (66) reported a 13% increase in peak power and total work performed during a maximal cycling bout of 30 s in healthy men and women who consumed 20 $g \cdot d^{-1}$ for 3 months, but who also reported increased activity during supplementation. There was not a significant effect of WHEY or CAS on GSH levels or performance in our subjects.

A positive correlation between NBAL and the change in postabsorptive cysteine levels from d 14 to d 21 was seen in CAS. This observation supports the conclusions of Droge and Holm (31) and Kinscherf et al. (62) that plasma cysteine levels modulate loss of lean tissue in certain conditions. These researchers suggest that sufficient free cysteine in the plasma is able to indirectly maintain levels of glutamine in times of increased urea production, reducing the need for protein catabolism for the release of glutamine. A connection between plasma cysteine and glutamine levels appears possible, as the change in postabsorptive cysteine was correlated with change in postabsorptive glutamine in WHEY in our study ($r = 0.743$, $p = 0.014$). However, whether any relationship between the two AA influences loss of body nitrogen is not evident, as although changes in cysteine correlated with cumulative NBAL in CAS, there was no correlation between changes in cysteine and glutamine in this group. The correlation between NBAL and plasma cysteine concentration in CAS but not in WHEY was unexpected, as WHEY consumed a much larger amount of cysteine throughout the experimental period. The two above mentioned findings separately suggest that plasma free cysteine level influences plasma glutamine concentration and that plasma cysteine also determines loss of body protein, but the presence of these observations in separate groups suggests that the two relationships are not strongly connected and that aspects of a hypothesis of this study and others (31, 62) are flawed.

Any number of explanations may account for this. While it is possible that cysteine concentrations influence glutamine levels, it could also be that plasma concentrations of other AA, such as BCAA, exercise a greater influence on proteolysis and loss of nitrogen. This would explain both the correlation between changes in cysteine and glutamine in WHEY, yet the lack of a correlation between levels of the AA and NBAL. Due to the large number of correlational analyses performed, it is also possible that the statistical significance of some of the observed correlations between plasma AA concentrations and NBAL may be due to chance. A third explanation takes into account other bodily AA pools. The plasma free AA pool accounts for a very small fraction of the total of AA in the body. While it is likely that plasma free AA levels exert some influence on loss of body protein, it is also possible that levels of peptides in the plasma, AA concentrations in other tissues or bound to elements in the blood, or

bioactivities exerted by products of digestion also have roles in the regulation of proteolysis and protein synthesis.

Correlations between BCAA concentration and cumulative NBAL were detected at 3 of the 4 time points for measurement of plasma AA in WHEY. The correlations observed in our study suggest that levels of plasma BCAA may influence loss of nitrogen during energy restriction in trained athletes. It is possible that high circulating levels of BCAA may inhibit proteolysis for the release of BCAA for use as energy in calorically restricted individuals. In a previous study, supplementation with BCAA was found to be associated with more positive NBAL than supplementation with other AA in healthy subjects undergoing bed rest (104). The whey protein supplement used in our study had approximately 15% more BCAA than did the casein. Although there was no effect of group on levels of BCAA in our study, others (46) have observed significantly greater increases in postprandial plasma free valine, isoleucine and leucine in subjects consuming a meal high in whey content versus those subjects receiving a casein meal. It is possible that the additional BCAA present in the supplement consumed by WHEY in our study were used preferentially as fuel and were thus unable to contribute to an increase in plasma free BCAA levels in this group. Furthermore, while changes in glutamine levels did not correlate with BCAA in either group in our study, other researchers (83) have observed that supplementing with BCAA prevented the decreases in plasma glutamine elicited by long-term exercise. This implicates BCAA in a proteolysis-regulating role similar to that proposed for cysteine by Droge and Holm (31), as a substrate used to regulate plasma free glutamine concentrations. These other studies, along with our data, suggest that sufficient levels of circulating BCAA may limit the catabolism of body protein for the release of free AA, including glutamine or BCAA.

Correlations in the levels of phenylalanine, ornithine and tryptophan with NBAL were seen on more than one occasion in our subjects. Levels of all three of these amino acids have been seen to be affected by wasting diseases such as cancer (118) and Crohn's disease (32). Phenylalanine negatively correlated with NBAL, which is not surprising, as levels have been shown to be indicative of protein breakdown (109). Changes in levels of phenylalanine have also been seen to negatively correlate with changes in BCM in subjects performing exercise (62). Gazzaniga et al. (42) observed a correlation between

changes in ornithine concentrations and NBAL in hospitalized adults receiving parenteral nutrition. Calbet and MacLean (22) include ornithine as one of a list of insulinergic amino acids. It is possible that levels of this amino acid may correlate to release of the anabolic hormone, insulin, which in turn improves protein retention.

Our study suggests that there is no difference in the value of the two investigated protein sources on loss of body nitrogen and maintenance of aerobic performance during weight loss. Although no differences were seen between groups in NBAL in our study, it remains a possibility that with a longer energy restriction period that differences may have become apparent. It is also possible that a longer period of supplementation, such as that by Lands et al. (66), may have made evident, differences in the proteins not elucidated with a 3 wk supplementation period. These considerations, along with others, are addressed in the following list of recommendations for future research.

Recommendations for Future Research

1. This study did not contain a control group with which to compare the effects of energy restriction on NBAL, performance and GSH when no supplemental protein is consumed and to determine plasma AA levels without consumption of a protein supplement. We observed increases in WBC GSH in both groups during energy restriction. A control group would bolster the argument that this increase is solely related to the low-energy diet and in no way to either or both of the proteins consumed during the diet. A control group would provide information on plasma AA concentrations, particularly postprandial concentrations, without the contribution of either protein. This would be particularly helpful for separating the effect on plasma AA of the protein and other macronutrients present in the formula diet from those of the additional protein supplement. A control group would also indicate any learning effect present in the measures of aerobic performance.

2. Due to limited availability of funds, plasma AA measurements were first taken on d 14, following 13 d of supplementation with either whey isolate or casein. It would have been valuable to have measured postabsorptive and postprandial plasma AA levels prior

to the beginning of supplementation, as with the data available it is impossible to know if chronic supplementation with 40 $g \cdot d^{-1}$ whey or casein during energy balance has an effect on fasted or postprandial plasma AA, or if AA levels prior to supplementation are related to NBAL.

3. As this study was conducted during the athletes' competitive season, most were unwilling to limit training or competition in the days immediately preceding an exercise test. As a result, exercise tests and GSH measurements were sometimes performed following competition on d 1, but following a day of rest or reduced training on d 15 and/or d 22. It would be valuable to repeat this experiment during the cycling/triathlon off-season so that subjects will be able to control training and report to the lab with similar levels of training induced soreness, fatigue, etc., increasing the reliability of performance data.

4. While HIV infected individuals have experienced significantly increased GSH from below-normal levels after 2 wk of supplementation with whey protein (77), healthy persons that experienced significant increases in GSH supplemented with whey for 3 months (66). Measurements were not made during the supplementation period in the latter study, so it is impossible to know when significant increases were first present. It is possible that our 17 d supplementation period preceding energy-restriction was not of sufficient duration to produce significant changes in GSH levels. A similar experiment employing a longer pre-diet supplementation period may be necessary to witness any GSH boosting tendencies of either of the protein supplements.

5. Most of our subjects reported that most, if not every day of the energy restriction period was a struggle. They were often hungry, lethargic and had difficulty concentrating. Many, although not all, reduced their training volume during this period. This elicits the question of whether this degree of energy restriction is realistic in a competitive, actively training population. It is likely that athletes attempting to lose weight but who would also like to maintain performance would choose a higher, yet still reduced, level of energy intake and longer period of restriction. A study employing

similar supplementation treatments but using such a modified energy restriction protocol, including typically consumed foods versus a liquid diet, may produce data and conclusions more applicable to the competitive athlete who is dieting to lose weight.

6. As Fruhbeck (39) reported, whey and casein have differing effects on levels of circulating postprandial glucagon and insulin, which in turn influence postprandial catabolism. Calbet and Maclean (22) suggest that levels of certain amino acids including lysine, ornithine, alanine, leucine, isoleucine, phenylalanine and arginine stimulate insulin secretion. Levels of several of these AA were found to correlate with NBAL at various time points (leucine and isoleucine positively; ornithine and phenylalanine both positively and negatively). It would be interesting to examine postprandial levels of these hormones over an extended period of time in our subjects to see if the different proteins – consumed as they were, with identical amounts of carbohydrate – did indeed have varying effects on hormone levels, how AA composition of each protein is related to hormonal response, and if postprandial levels of these hormones are related to NBAL. Along these same lines, it would also be valuable to perform additional measures of plasma AA for an extended period following meal consumption to observe how levels of these AA change over time postprandially and if these changes are related to hormone levels.

7. The measure of lean tissue loss employed in this study was an estimate of apparent nitrogen balance. Several assumptions were made when making this estimation: 1) dermal nitrogen loss was 5.5 mg $N \cdot kg^{-1} \cdot d^{-1}$, 2) protein supplements were 100% digestible, and 3) Ensure® was 94.2% digestible. A more complete analysis of nitrogen balance would include the very labor-intensive and unpleasant measurement of fecal and dermal nitrogen losses. To attempt to regulate dermal losses (as well as other variables), future studies could control for equal training in all subjects as different amounts of and environments for training lead to varying dermal nitrogen losses (63). An alternative to nitrogen balance for assessing protein status could be the use of labeled leucine for the comparison of protein synthesis and breakdown between groups.

8. This study only involved men. Some have suggested that active female endurance athletes may experience less negative nitrogen balance than men participating in exercise and consuming 0.86 $g \cdot kg^{-1} \cdot d^{-1}$ PRO (84). Additionally, the effect of whey supplementation on NBAL, GSH and free AA concentrations in men and women may not be identical, as dissimilarities in male and female physiology include differing glutathione peroxidase activity (92) and plasma AA levels (5). Until similar investigations have been conducted involving women, the ability of whey protein isolate to improve NBAL and performance and to enhance blood GSH in competitive young athletes cannot be totally discounted.

9. Our subjects were instructed to consume their protein supplements once in the morning and once in the evening. This may not have been the optimal consumption pattern for protein accretion and avoidance of proteolysis, particularly during energy restriction. Work in individuals undergoing resistance training has shown that amino acid infusion (10) and oral amino acids (105) increase protein synthesis and may attenuate protein breakdown following exercise. Roy et al. (93) report that a mixed macronutrient beverage following endurance exercise improved nitrogen balance relative to placebo. As Boirie et al. (13) described, whey and casein proteins are digested at different rates, resulting in varying appearance times for free AA in the plasma and influencing protein accretion. Coordinating feeding times in order to strategically enduce hyperaminoacidemia in these subjects may elicit benefits to protein retention not encountered with our general ingestion instructions.

10. Recent research suggests that the opioid activities possessed by whey and casein may include influence on satiety (46). These researchers observed greater plasma cholycystokinin concentrations and greater reported satiety with a whey "pre-load" compared to casein. Other researchers suggest that properties of whey may help achieve loss of adipose tissue with supplementation (44). While our research does not suggest superiority of whey for lean tissue preservation while dieting, it is possible that the benefit of whey protein may lie in its ability to encourage satiety, maintaining elective

energy restriction and, possibly, elicit fat loss through an as-of-yet unelucidated mechanism.

11. Despite essentially identical amounts of tryptophan in the whey protein isolate and casein used in this study, WHEY experienced a greater increase of this AA in the plasma 2 h following meal ingestion. High levels of circulating tryptophan are hypothesized to hasten the onset of central fatigue, as the availability of this AA is thought to regulate the synthesis of 5-hydroxytryptamine in brain (11). As our subjects did not consume protein supplements before each exercise test, levels of tryptophan are not thought to have affected performance in this study. However, the presence of significantly higher levels of tryptophan following whey ingestion in our subjects suggests that consumption of whey protein isolate before exercise may be detrimental to performance, relative to casein consumption. Investigation of this hypothesis is warranted.

12. While some of our data suggest that levels of plasma cysteine may somehow influence loss of body nitrogen, it is unclear whether the original hypothesized mechanism for plasma free cysteine and lean tissue loss put forth by Droge and Holm (31) applies to well-trained aerobic athletes undergoing energy restriction. Critical to their theory is an increase in urea production which elicits decreases in plasma free cysteine levels as the AA is broken down in liver for the release of protons. While the training schedules and energy deficit that our subjects endured are likely to have increased urea production, we cannot be sure of the extent to which this occurred. Additional hematological measures performed at baseline, prior to energy restriction and at its completion would have provided information concerning the effects of the experimental interventions on urea concentration. It is possible that more extreme stressors and larger increases in urea production are necessary to dysregulate Droge and Holm's "postabsorptive glutamate/cystine shuttle" in a healthy and well-trained population.

13. Employing mRNA microarray and proteomic technology in the analysis of samples taken throughout the supplementation, energy restriction and acute meal ingestion

periods would provide much valuable data concerning the influence of various biochemical factors on the metabolism of this population. Although our data do not support the conclusion that supplementation with whey protein isolate or casein prior to and during energy restriction differently affects NBAL, it is possible that characteristics of these proteins may have led to different mechanisms for the modulation of body protein metabolism during supplementation. For example, biologically active peptides in whey may have caused the down-regulation of the synthesis of proteolytic enzymes, while casein proteins may have acted to up-regulate enzymes critical in body protein synthesis. Additionally, others have reported levels of plasma AA to modulate gene expression (60). While, in practice, supplementing with either of these proteins may not prove beneficial to dieting athletes, investigation into the biochemical effects of supplementation on this population may generate data applicable and valuable in other contexts.

Appendix A: Detailed Description of Research Methods and Procedures

Subject Selection and Screening

Twenty-one males between the ages of 19 and 29 years were selected for the experimental study. One subject withdrew from the study due to an adverse reaction to blood draw procedures. Most subjects were well-trained with nearly all actively competing in collegiate cycling and/or triathlon contests. Subjects had not consumed any whey protein-containing nutritional supplements for at least one month prior to the beginning of the experimental period and refrained from taking any nutritional supplements during the experimental period.

Subjects were invited to group information sessions to hear details of participation and potential risks. Subjects completed a health history questionnaire to ensure they met the criteria of the American College of Sports Medicine (ACSM) for "low risk" for exercise participation and testing. Additional exclusionary criteria related to the low calorie diet and supplement consumption included eating disorders and food allergies. Subjects gave their informed consent and the study was approved by the Institutional Review Board.

Subject Pre-Testing

Baseline measurements of body composition and aerobic fitness were performed on all subjects no more than 2 wk prior to the beginning of the experimental study. Body composition was determined using a three-site skin fold equation derived by Jackson and Pollock (55). The same trained technician performed all skin fold measurements at the abdomen, suprailliac and tricep.

Peak oxygen consumption (VO_{2peak}) was determined for all subjects using a graded exercise test on a Sensormedics 800 cycle ergometer (Sensormedics, Yorba Linda, CA). Prior to VO_{2peak} testing, subjects were familiarized with exercise on the ergometer at a constant pedaling rate while breathing through a mouthpiece and breathing valve. The test began at a low intensity, 100 watts, with subjects pedaling at a preferred cadence, and increased 15 watts every 30 s until the subject could no longer maintain 60 rpm or reached volitional exhaustion. Continuous indirect calorimetry was performed during the test using a Sensormedics Vmax 229 metabolic cart system (Sensormedics). Resistance, perceived effort and oxygen consumption were recorded every 30 s. The

wattage found to elicit 55%, 70% and 90% of VO_{2peak} during the peak oxygen consumption test was calculated and used as resistance during the submaximal exercise and time-to-exhaustion tests.

Subjects had measurements of body weight taken during pre-testing, as well as prior to submaximal exercise testing on d 1, 15 and 22 of the experimental period.

Submaximal Exercise and Time-to-Exhaustion Tests

Subjects reported to the lab on the mornings of d 1, 15 and 22 of the experimental period, 1 h after consuming a standardized breakfast of one can (237 mL/250 kcal) of Ensure® formula diet (Abbott Laboratories, Abbott Park, IL) (63.9% CHO, 14.1% PRO, 22% Fat) and no other food or drink save water. Subjects were positioned on the cycle ergometer and allowed to warm-up with a resistance of 100 watts for 5 min. Immediately following the warm-up period, subjects began a submaximal exercise bout entailing 7 min at a resistance that elicted 70% VO_{2peak} followed by 38 min at a resistance associated with 55% VO_{2peak}. Oxygen consumption was measured using a Sensormedics Vmax 229 metabolic cart once during the 70% phase and twice during the 55% phase.

At the completion of the 45 min submaximal exercise test, subjects stopped pedaling for approximately 1 min while blood samples were taken. Subjects then immediately began a timed test to exhaustion at 90% VO_{2peak} until they could no longer maintain 60 rpm or until volitional exhaustion.

Protein Supplementation and Caloric Restriction

Subjects were randomly placed in either the whey protein isolate (WHEY) or casein group (CAS). Both experimenters and subjects were blinded to group membership. From d 1 to d 17 of the experimental period, in addition to their normal diet, subjects consumed $40 g \cdot d^{-1}$ of protein corresponding to group placement. Subjects were given 1 wk supplies of protein on d 1 and 8 that consisted of 14 bags each containing 20 g of protein which had been weighed out by the experimenters, and a 3 d supply (6 bags) on d 15, and instructed to consume one bag of protein in the morning and one in the evening daily. On d 1, subjects received instructions concerning method of protein consumption, which included a warning to avoid blending the protein

supplements or mixing the supplements in hot foods or liquids. Subjects were also instructed to consume the supplements no more than 30 min following mixing of the protein in a food or beverage. At the end of each week, subjects returned a compliance form detailing patterns of protein supplement consumption.

On d 18, 19, 20 and 21 subjects consumed 40 $g \cdot d^{-1}$ of protein in addition to 20 $kcal \cdot kg^{-1} \cdot d^{-1}$ of a formula diet (Ensure®). Subjects came to the lab each morning and received a 1 d supply of formula diet and protein.

Urine Samples and Analysis

Subjects completed 24 h urine collections during each of the 4 d of the energy restriction period. Samples were collected in 1 L polypropylene bottles with 2 mL 50% hydrochloric acid to ensure that ammonia did not become volatile. Collections began with the second void of the day and were brought to the lab after the first void of the next morning. The samples were well mixed and total volume was recorded. Aliquots were frozen for later analysis of nitrogen and creatinine.

On the days in which urine analyses were performed, urine samples were thawed at room temperature and well mixed. Creatinine concentration was analyzed in duplicate spectrophotometrically according to directions from a commercially available kit employing picric acid reagent (Creatinine Procedure No. 0400, Stanbio Laboratory, Boerne, TX). Duplicates with greater than 10% difference were reanalyzed. Urinary nitrogen was analyzed in duplicate using the well-known Kjeldahl technique involving digestion with sulfuric acid, distillation of ammonia and titration with boric acid, on a Buchi AG, B-339 distillation apparatus. Duplicates with greater than 7% difference were reanalyzed.

Apparent NBAL was calculated for each day of the energy restriction period separately. Dietary nitrogen was calculated using the manufacturer's data for nitrogen content of the formula diet, while nitrogen contents of the protein supplements were analyzed by the experimenters using the Kjeldahl technique and found to contain 13% nitrogen by mass. Fecal nitrogen was estimated using the protein digestibility value for the formula diet provided by the manufacturer (Abbott Laboratories) -- 94.2% digested, 5.8% lost in feces. Given the milk source of the protein supplements, they were assumed

to have essentially 100% digestibility and thus not contribute to fecal nitrogen. Dermal nitrogen loss was estimated using the value established by Rand et al. (88), of 5.5 mg $N \cdot kg^{-1} \cdot d^{-1}$ for temperate climates. Cumulative NBAL was calculated as the sum of individual NBAL values for d 18, 19, 20 and 21.

Blood Collection and Analysis

On the mornings of d 14 and 21, following an overnight fast of at least 10 h, subjects reported to the lab and blood samples were taken from an arm vein immediately prior to and 2 h following consumption of one can of Ensure® (237 mL/250 kcal) mixed with 20 g of protein supplement, to determine the effects of caloric restriction on postabsorptive and postprandial plasma AA concentrations. Blood samples were immediately spun at 1500 X g for 15 min at 4ºC. Plasma was immediately removed after centrifugation and thoroughly mixed with 25.6 mg/L norleucine in methanol in a 1:2 ratio, plasma to methanol. The plasma-methanol mixture was capped, refrigerated overnight at 4ºC to allow the solution to deproteinize and then spun at 27000 X g for 20 min at 4ºC. The supernatant was decanted, purged with nitrogen gas and stored at -20ºC for later analysis of plasma amino acids. Plasma amino acids were analyzed in duplicate by amino acid analyzer (PICO.TAG, Waters Association, Milford, MA) in the laboratory of Dr. Ken Webb of the Virginia Tech Department of Animal and Poultry Sciences, according to a procedure published by Bidlingmeyer et al. (8).

Blood samples for the analysis of GSH were collected on d 1, 15 and 22 immediately before beginning the submaximal exercise test. White blood cells were separated from one sample of whole blood using Vacutainer Cell Preparation Tubes (Vacutainer CPT, Becton Dickinson and Co., Franklin Lakes, NJ). After washing with PBS, WBC were resuspended in 200 µl PBS and counted using a Coulter Counter (Beckman Coulter, Inc., Fullerton, CA). Samples of both whole blood and WBC samples were frozen at -70º C for future analysis. Both whole blood and WBC GSH were measured using a commercially available spectrophotometric assay (GSH/GSSG-412 assay, OxisResearch, Portland, OR). Whole blood was analyzed for total protein using a detergent free protein assay (Bio-Rad Laboratories, Hercules, CA) in order to report GSH per mg protein.

Appendix B: Individual Values for Lab Measurements

Table 1. Raw Data for Descriptives

Subject	Group	Age (yrs)	Height (in)	VO2peak (L/kg/min)	Body Fat %	Wt (kg) d 1	Wt (kg) d 15	Wt (kg) d 21
1	WHEY	20	170.2	63.5	9.1	65.1	65.0	62.5
3	WHEY	21	175.3	49.4	8.3	81.4	82.5	80.0
4	WHEY	20	192.4	54.1	18.4	84.3	84.5	81.5
9	WHEY	25	174.0	73.6	4.9	66.0	65.9	64.0
11	WHEY	20	162.5	56.4	10.4	59.9	60.0	58.5
12	WHEY	21	172.7	66.0	14.7	69.7	68.2	66.3
13	WHEY	19	166.4	68.1	6.0	65.2	64.5	61.7
15	WHEY	23	180.3	60.0	8.0	76.7	73.7	73.6
18	WHEY	24	184.2	58.7	11.8	83.1	85.4	80.0
20	WHEY	24	175.3	57.2	8.4	68.5	69.6	66.6
	Mean	**21.7**	**175.3**	**60.7**	**10.0**	**72.0**	**71.9**	**69.5**
	S.E.	**0.7**	**2.7**	**2.3**	**1.3**	**2.8**	**2.9**	**2.7**

Subject	Group	Age (yrs)	Height (in)	VO2peak (L/kg/min)	Body Fat %	Wt (kg) d 1	Wt (kg) d 15	Wt (kg) d 21
2	CAS	23	176.5	48.6	16.0	76.3	76.1	74.2
5	CAS	19	177.8	53.7	9.6	68.0	69.1	64.1
6	CAS	29	167.6	55.7	10.0	58.2	59.2	57.4
7	CAS	23	184.2	62.7	7.9	75.2	76.5	72.2
8	CAS	25	187.9	68.5	5.3	79.7	79.6	77.5
10	CAS	23	180.3	43.8	18.4	85.6	88.6	85.7
14	CAS	23	175.3	57.5	8.9	67.5	67.7	64.8
16	CAS	28	186.7	51.4	11.6	76.7	76.0	73.6
17	CAS	22	164.5	63.9	9.8	67.5	69.0	66.6
21	CAS	19	185.4	57.1	11.2	75.5	76.6	73.5
	Mean	**23.4**	**178.6**	**56.3**	**11.4**	**73.0**	**73.8**	**71.0**
	S.E.	**1.0**	**2.5**	**2.4**	**1.1**	**2.5**	**2.5**	**2.5**

Table 2. Raw Data for Time-to-Exhaustion (s)

Subject	Group	TTE (s) d 1	TTE (s) d 15	TTE (s) d 21
1	WHEY	*	72	128
3	WHEY	248	276	98
4	WHEY	274	123	62
9	WHEY	309	258	151
11	WHEY	124	244	241
12	WHEY	370	271	360
13	WHEY	155	222	98
15	WHEY	219	*	251
18	WHEY	320	316	272
20	WHEY	212	*	272
	Mean	**247.9**	**222.8**	**193.3**
	S.E.	**26.5**	**29.4**	**31.1**

Subject	Group	TTE (s) d 1	TTE (s) d 15	TTE (s) d 21
2	CAS	200	225	175
5	CAS	128	301	422
6	CAS	76	*	121
7	CAS	105	331	128
8	CAS	254	310	420
10	CAS	124	200	129
14	CAS	222	252	216
16	CAS	246	363	146
17	CAS	192	307	184
21	CAS	146	161	106
	Mean	**169.3**	**272.2**	**204.7**
	S.E.	**19.6**	**22.2**	**37.5**

* indicates absence of data due to procedural error

Table 3. Raw Data for Urinary and Dietary Nitrogen Content (g)

Subject	Group	Urinary N (g) d 18	Urinary N (g) d 19	Urinary N (g) d 20	Urinary N (g) d 21	Formula Diet N (g) d 18	Formula Diet N (g) d 19	Formula Diet N (g) d 20	Formula Diet N (g) d 21
1	WHEY	17.201	19.856	16.413	17.511	7.331	7.331	7.331	7.331
3	WHEY	16.883	22.57	18.335	17.963	9.238	9.238	9.238	9.238
4	WHEY	23.092	18.257	18.253	19.42	9.767	9.767	9.767	9.767
9	WHEY	21.574	18.267	18.225	20.957	7.503	7.503	7.503	7.503
11	WHEY	14.947	13.442	11.081	13.743	6.565	6.565	6.565	6.565
12	WHEY	14.660	13.371	14.504	16.054	8.062	8.062	8.062	8.062
13	WHEY	18.328	16.362	18.522	16.692	7.206	7.206	7.206	7.206
15	WHEY	19.054	15.801	16.776	19.868	8.448	8.448	8.448	8.662
18	WHEY	19.495	21.633	23.544	24.465	8.050	9.458	9.458	9.458
20	WHEY	18.420	17.539	13.384	17.008	7.836	7.836	7.836	7.836
	Mean	**18.37**	**17.71**	**16.9**	**18.37**	**8.00**	**8.14**	**8.14**	**8.16**
	S.E.	**0.84**	**0.98**	**1.08**	**0.94**	**0.30**	**0.34**	**0.34**	**0.34**

Subject	Group	Urinary N (g) d 18	Urinary N (g) d 19	Urinary N (g) d 20	Urinary N (g) d 21	Formula Diet N (g) d 18	Formula Diet N (g) d 19	Formula Diet N (g) d 20	Formula Diet N (g) d 21
2	CAS	16.605	15.149	18.286	16.855	8.513	8.644	8.448	8.448
5	CAS	14.920	15.355	16.578	16.465	7.545	7.545	7.545	7.545
6	CAS	16.899	13.747	14.607	14.157	6.589	6.589	6.589	6.589
7	CAS	20.090	17.444	17.107	19.019	8.448	8.448	8.448	8.543
8	CAS	24.040	19.553	17.482	20.543	8.739	8.739	8.739	8.739
10	CAS	20.900	20.286	16.924	19.536	9.654	9.654	9.654	9.654
14	CAS	20.032	14.312	16.722	17.942	7.545	7.545	7.545	7.545
16	CAS	19.820	16.419	16.606	17.820	8.448	8.448	8.448	8.852
17	CAS	19.332	17.407	16.695	14.812	7.658	7.658	7.658	7.658
21	CAS	17.973	16.705	15.582	17.992	8.614	8.614	8.614	8.614
	Mean	**19.06**	**16.64**	**16.66**	**17.51**	**8.18**	**8.19**	**8.17**	**8.22**
	S.E.	**0.82**	**0.67**	**0.32**	**0.63**	**0.27**	**0.27**	**0.27**	**0.28**

Table 4. Raw Data for Estimated Fecal and Dermal Nitrogen Losses (g)

Subject	Group	Est. Fecal N (g) d 18	Est. Fecal N (g) d 19	Est. Fecal N (g) d 20	Est. Fecal N (g) d 21	Est. Dermal N (g) d 18	Est. Dermal N (g) d 19	Est. Dermal N (g) d 20	Est. Dermal N (g) d 21
1	WHEY	0.425	0.425	0.425	0.425	0.326	0.326	0.326	0.326
3	WHEY	0.536	0.536	0.536	0.536	0.407	0.407	0.407	0.407
4	WHEY	0.567	0.567	0.567	0.567	0.422	0.422	0.422	0.422
9	WHEY	0.435	0.435	0.435	0.435	0.330	0.330	0.330	0.330
11	WHEY	0.381	0.381	0.381	0.381	0.300	0.300	0.300	0.300
12	WHEY	0.468	0.468	0.468	0.468	0.349	0.349	0.349	0.349
13	WHEY	0.418	0.418	0.418	0.418	0.326	0.326	0.326	0.326
15	WHEY	0.490	0.490	0.490	0.502	0.384	0.384	0.384	0.384
18	WHEY	0.467	0.549	0.549	0.549	0.416	0.416	0.416	0.416
20	WHEY	0.455	0.455	0.455	0.455	0.343	0.343	0.343	0.343
	Mean	**0.464**	**0.472**	**0.472**	**0.473**	**0.36**	**0.36**	**0.36**	**0.36**
	S.E.	**0.018**	**0.02**	**0..02**	**0.02**	**0.014**	**0.014**	**0.014**	**0.014**

Subject	Group	Est. Fecal N (g) d 18	Est. Fecal N (g) d 19	Est. Fecal N (g) d 20	Est. Fecal N (g) d 21	Est. Dermal N (g) d 18	Est. Dermal N (g) d 19	Est. Dermal N (g) d 20	Est. Dermal N (g) d 21
2	CAS	0.494	0.501	0.490	0.490	0.382	0.382	0.382	0.382
5	CAS	0.438	0.438	0.438	0.438	0.340	0.340	0.340	0.340
6	CAS	0.382	0.382	0.382	0.382	0.291	0.291	0.291	0.291
7	CAS	0.490	0.490	0.490	0.496	0.376	0.376	0.376	0.376
8	CAS	0.507	0.507	0.507	0.507	0.399	0.399	0.399	0.399
10	CAS	0.560	0.560	0.560	0.560	0.428	0.428	0.428	0.428
14	CAS	0.438	0.438	0.438	0.438	0.338	0.338	0.338	0.338
16	CAS	0.490	0.490	0.490	0.513	0.384	0.384	0.384	0.384
17	CAS	0.444	0.444	0.444	0.444	0.338	0.338	0.338	0.338
21	CAS	0.500	0.500	0.500	0.500	0.378	0.378	0.378	0.378
	Mean	**0.474**	**0.475**	**0.474**	**0.477**	**0.365**	**0.365**	**0.365**	**0.365**
	S.E.	**0.016**	**0.016**	**0.016**	**0.016**	**0.012**	**0.012**	**0.012**	**0.012**

Table 5. Raw Data for Urinary Creatinine

Subject	Group	Urinary Creatinine (g/dL) d 18	Urinary Creatinine (g/dL) d 19	Urinary Creatinine (g/dL) d 20	Urinary Creatinine (g/dL) d 21	Creatinine (g/24h) d 18	Creatinine (g/24h) d 19	Creatinine (g/24h) d 20	Creatinine (g/24h) d 21
1	WHEY	0.048	0.048	0.069	0.156	1.637	1.908	1.520	1.665
3	WHEY	0.054	0.077	0.075	0.067	1.806	2.544	2.040	2.005
4	WHEY	0.050	0.074	0.059	0.099	2.110	2.207	2.152	2.299
9	WHEY	0.068	0.056	0.045	0.052	1.830	1.892	1.825	2.055
11	WHEY	0.038	0.038	0.088	0.053	1.586	1.523	1.349	1.409
12	WHEY	0.056	0.049	0.073	0.070	1.630	1.521	1.726	1.816
13	WHEY	0.032	0.033	0.036	0.037	1.615	1.607	1.810	1.647
15	WHEY	0.021	0.017	0.020	0.023	1.936	1.586	1.946	2.335
18	WHEY	0.051	0.058	0.055	0.058	1.891	1.912	1.913	1.897
20	WHEY	0.031	0.029	0.039	0.043	1.778	1.907	1.540	1.989
	Mean	**0.045**	**0.048**	**0.056**	**0.066**	**1.782**	**1.861**	**1.782**	**1.912**
	S.E.	**0.005**	**0.006**	**0.007**	**0.019**	**0.053**	**0.103**	**0.079**	**0.092**

Subject	Group	Urinary Creatinine (g/dL) d 18	Urinary Creatinine (g/dL) d 19	Urinary Creatinine (g/dL) d 20	Urinary Creatinine (g/dL) d 21	Creatinine (g/24h) d 18	Creatinine (g/24h) d 19	Creatinine (g/24h) d 20	Creatinine (g/24h) d 21
2	CAS	0.044	0.049	0.046	0.060	2.124	1.92	2.203	2.061
5	CAS	0.043	0.111	0.059	0.060	1.412	1.469	1.330	1.571
6	CAS	0.074	0.167	0.081	0.073	1.657	1.620	1.514	1.461
7	CAS	0.052	0.092	0.043	0.056	1.965	1.879	1.506	1.950
8	CAS	0.030	0.025	0.023	0.026	2.223	1.901	1.793	2.024
10	CAS	0.077	0.046	0.059	0.045	2.295	2.132	1.712	2.198
14	CAS	0.054	0.092	0.084	0.075	1.924	1.685	1.865	1.896
16	CAS	0.038	0.049	0.054	0.066	1.696	1.640	1.504	1.723
17	CAS	0.038	0.043	0.037	0.030	1.782	1.718	1.725	1.527
21	CAS	0.044	0.044	0.042	0.044	1.879	1.754	1.560	1.799
	Mean	**0.049**	**0.072**	**0.053**	**0.053**	**1.896**	**1.772**	**1.671**	**1.821**
	S.E	**0.005**	**0.014**	**0.006**	**0.005**	**0.086**	**0.060**	**0.078**	**0.078**

Table 6. Raw Data for Urinary Nitrogen/Creatinine and Daily Nitrogen Balance

Subject	Group	Urinary N (g)/Creatinine (g) d 18	Urinary N (g)/Creatinine (g) d 19	Urinary N (g)/Creatinine (g) d 20	Urinary N (g)/Creatinine (g) d 21	Nitrogen Balance (g) d 18	Nitrogen Balance (g) d 19	Nitrogen Balance (g) d 20	Nitrogen Balance (g) d 21
1	WHEY	10.510	10.406	10.797	10.519	-5.421	-8.076	-4.633	-5.73
3	WHEY	9.346	8.872	8.988	8.958	-3.388	-9.075	-4.840	-4.467
4	WHEY	10.942	8.273	8.484	8.447	-9.114	-4.278	-4.274	-5.441
9	WHEY	11.791	9.655	9.985	10.200	-9.635	-6.328	-6.287	-9.019
11	WHEY	9.425	8.825	8.213	9.755	-3.863	-2.357	0.003	-2.659
12	WHEY	8.997	8.791	8.404	8.838	-2.214	-0.925	-2.058	-3.608
13	WHEY	11.350	10.180	10.232	10.137	-6.666	-4.699	-6.859	-5.029
15	WHEY	9.840	9.963	8.622	8.510	-6.280	-3.027	-4.001	-6.892
18	WHEY	10.311	11.313	12.306	12.898	-7.128	-7.939	-9.850	-10.771
20	WHEY	10.361	9.197	8.692	8.553	-6.181	-5.300	-1.145	-4.769
	Mean	**10.29**	**9.547**	**9.472**	**9.681**	**-5.989**	**-5.200**	**-4.390**	**-5.838**
	S.E.	**0.286**	**0.293**	**0.420**	**0.434**	**0.751**	**0.845**	**0.914**	**0.778**

Subject	Group	Urinary N (g)/Creatinine (g) d 18	Urinary N (g)/Creatinine (g) d 19	Urinary N (g)/Creatinine (g) d 20	Urinary N (g)/Creatinine (g) d 21	Nitrogen Balance (g) d 18	Nitrogen Balance (g) d 19	Nitrogen Balance (g) d 20	Nitrogen Balance (g) d 21
2	CAS	7.820	7.889	8.302	8.178	-3.767	-2.188	-5.510	-4.078
5	CAS	10.570	10.451	12.469	10.478	-2.953	-3.387	-4.611	-4.497
6	CAS	10.199	8.487	9.648	9.690	-5.784	-2.631	-3.492	-3.041
7	CAS	10.223	9.285	11.362	9.754	-7.308	-4.662	-4.325	-6.147
8	CAS	10.814	10.287	9.751	10.151	-11.006	-6.519	-4.448	-7.509
10	CAS	9.109	9.516	9.887	8.887	-7.034	-6.420	-3.058	-5.669
14	CAS	10.409	8.496	8.969	9.464	-8.062	-2.342	-4.752	-5.972
16	CAS	11.687	10.015	11.038	10.343	-7.045	-3.645	-3.831	-4.665
17	CAS	10.846	10.130	9.679	9.698	-7.256	-5.331	-4.619	-2.736
21	CAS	9.567	9.527	9.99	10.003	-5.036	-3.768	-2.645	-5.055
	Mean	**10.124**	**9.408**	**10.109**	**9.664**	**-6.525**	**-4.089**	**-4.129**	**-4.937**
	S.E.	**0.340**	**0.274**	**0.382**	**0.220**	**0.725**	**0.503**	**0.274**	**0.461**

Table 7. Raw Data for Glutamic Acid and Serine (μM)

Subject	Group	Glutamic Acid (μM) d 14 PA	Glutamic Acid (μM) d 14 PP	Glutamic Acid (μM) d 21 PA	Glutamic Acid (μM) d 21 PP	Serine (μM) d 14 PA	Serine (μM) d 14 PP	Serine (μM) d 21 PA	Serine (μM) d 21 PP
1	WHEY	18.98	33.15	21.15	20.29	84.85	128.82	115.96	129.51
3	WHEY	31.06	52.90	34.08	46.63	65.90	64.75	89.78	81.63
4	WHEY	54.14	43.30	37.10	37.41	93.46	100.69	87.94	95.75
9	WHEY	29.12	27.42	19.21	18.05	92.65	91.85	89.21	69.92
11	WHEY	28.66	40.82	23.39	26.65	95.87	111.48	113.32	115.38
12	WHEY	16.19	21.77	19.52	23.32	99.20	110.33	127.67	129.28
13	WHEY	23.39	27.81	23.47	32.53	80.60	94.14	98.05	78.87
15	WHEY	39.35	26.18	23.55	24.09	107.69	121.24	109.99	119.29
18	WHEY	19.60	20.45	21.84	18.67	91.16	94.72	104.25	86.80
20	WHEY	29.12	25.64	26.34	25.48	77.04	90.47	101.26	108.27
	Mean	**28.96**	**31.94**	**24.97**	**27.31**	**88.84**	**100.85**	**103.74**	**101.47**
	S.E.	**3.54**	**3.32**	**1.90**	**2.87**	**3.80**	**5.75**	**4.15**	**6.88**

Subject	Group	Glutamic Acid (μM) d 14 PA	Glutamic Acid (μM) d 14 PP	Glutamic Acid (μM) d 21 PA	Glutamic Acid (μM) d 21 PP	Serine (μM) d 14 PA	Serine (μM) d 14 PP	Serine (μM) d 21 PA	Serine (μM) d 21 PP
2	CAS	20.91	27.19	25.10	23.63	105.17	114.58	113.89	142.14
5	CAS	12.78	16.65	21.46	21.77	115.15	110.10	99.43	82.78
6	CAS	25.41	28.12	18.90	23.70	121.93	131.92	127.90	133.30
7	CAS	29.05	29.36	16.89	16.89	112.74	128.47	107.00	116.30
8	CAS	20.91	23.39	15.96	24.94	87.26	104.94	109.76	129.97
10	CAS	41.21	33.69	24.86	25.48	119.06	126.75	107.92	99.66
14	CAS	49.50	52.83	32.38	58.79	119.63	143.51	132.49	163.03
16	CAS	22.62	30.60	21.77	27.96	88.98	111.48	90.82	101.03
17	CAS	17.66	21.61	14.72	26.10	82.32	109.87	93.80	120.32
21	CAS	28.27	29.12	15.49	26.57	89.44	111.60	101.84	127.44
	Mean	**26.83**	**29.26**	**20.75**	**27.58**	**104.17**	**119.32**	**108.48**	**121.60**
	S.E.	**3.49**	**3.04**	**1.76**	**3.60**	**4.92**	**3.95**	**4.27**	**7.32**

Table 8. Raw Data for Asparagine and Glycine (µM)

Subject	Group	Asparagine (µM) d 14 PA	Asparagine (µM) d 14 PP	Asparagine (µM) d 21 PA	Asparagine (µM) d 21 PP	Glycine (µM) d 14 PA	Glycine (µM) d 14 PP	Glycine (µM) d 21 PA	Glycine (µM) d 21 PP
1	WHEY	77.38	138.90	97.36	130.05	258.32	305.95	321.89	318.74
3	WHEY	68.79	74.05	89.48	85.71	240.81	221.89	285.99	236.95
4	WHEY	82.81	95.35	81.68	90.26	379.16	356.74	290.89	282.49
9	WHEY	78.17	83.78	83.52	69.58	315.06	274.08	266.55	206.13
11	WHEY	88.25	108.32	101.48	111.91	256.22	236.60	266.02	231.00
12	WHEY	79.40	94.38	93.59	107.62	342.73	340.11	374.96	344.13
13	WHEY	114.89	120.76	107.18	91.05	287.04	247.11	301.05	224.87
15	WHEY	117.61	135.83	105.51	117.17	312.96	306.48	332.4	397.02
18	WHEY	113.49	121.90	118.04	111.82	362.17	304.90	310.16	228.90
20	WHEY	90.96	109.02	102.80	116.99	230.30	214.36	277.76	245.01
	Mean	**91.18**	**108.23**	**98.06**	**103.22**	**298.48**	**280.82**	**302.77**	**271.52**
	S.E.	**5.61**	**6.81**	**3.57**	**5.80**	**16.50**	**15.70**	**10.66**	**19.77**

Subject	Group	Asparagine (µM) d 14 PA	Asparagine (µM) d 14 PP	Asparagine (µM) d 21 PA	Asparagine (µM) d 21 PP	Glycine (µM) d 14 PA	Glycine (µM) d 14 PP	Glycine (µM) d 21 PA	Glycine (µM) d 21 PP
2	CAS	127.60	123.74	139.08	149.50	312.08	352.89	328.72	354.47
5	CAS	104.55	104.55	94.21	87.46	292.29	248.51	271.10	203.68
6	CAS	90.09	100.87	95.00	103.32	445.01	434.15	452.01	436.60
7	CAS	113.57	125.76	109.54	125.05	293.35	282.66	263.05	256.74
8	CAS	94.03	103.32	100.34	118.22	309.63	306.48	321.19	306.30
10	CAS	110.33	120.41	101.22	111.47	273.91	257.97	234.68	243.96
14	CAS	109.72	131.63	109.28	143.98	353.94	369.35	325.57	353.42
16	CAS	86.58	101.66	84.13	92.10	292.82	287.04	310.86	267.60
17	CAS	90.79	126.02	92.63	129.35	361.47	296.15	291.42	304.73
21	CAS	63.10	88.95	72.47	101.48	201.40	223.47	208.93	226.62
	Mean	**99.04**	**112.69**	**99.79**	**116.19**	**313.59**	**305.87**	**300.75**	**295.41**
	S.E.	**5.70**	**4.56**	**5.61**	**6.62**	**20.18**	**20.02**	**21.08**	**22.37**

Table 9. Raw Data for Glutamine and Taurine (µM)

Subject	Group	Glutamine (µM) d 14 PA	Glutamine (µM) d 14 PP	Glutamine (µM) d 21 PA	Glutamine (µM) d 21 PP	Taurine (µM) d 14 PA	Taurine (µM) d 14 PP	Taurine (µM) d 21 PA	Taurine (µM) d 21 PP
1	WHEY	567.45	686.89	702.19	710.07	46.97	51.91	51.63	44.35
3	WHEY	605.70	561.67	650.51	556.21	103.92	105.23	106.16	109.90
4	WHEY	702.58	727.01	662.53	683.92	64.89	64.61	83.29	52.10
9	WHEY	741.69	710.93	731.54	605.46	64.33	57.24	51.91	49.30
11	WHEY	706.64	700.47	704.76	667.68	46.22	40.52	48.55	41.55
12	WHEY	753.40	737.16	796.64	734.82	66.85	85.99	65.64	59.57
13	WHEY	722.64	699.61	699.92	576.81	38.10	34.08	48.65	39.03
15	WHEY	731.77	729.66	647.78	649.49	64.89	63.21	63.40	74.79
18	WHEY	858.86	769.63	835.75	733.72	51.07	42.02	56.02	54.53
20	WHEY	575.49	598.28	612.10	656.83	77.12	55.09	63.12	63.49
	Mean	**696.62**	**692.13**	**704.37**	**657.50**	**62.44**	**59.99**	**63.84**	**58.86**
	S.E.	**28.53**	**20.27**	**21.83**	**19.69**	**5.96**	**6.85**	**5.76**	**6.62**

Subject	Group	Glutamine (µM) d 14 PA	Glutamine (µM) d 14 PP	Glutamine (µM) d 21 PA	Glutamine (µM) d 21 PP	Taurine (µM) d 14 PA	Taurine (µM) d 14 PP	Taurine (µM) d 21 PA	Taurine (µM) d 21 PP
2	CAS	713.19	729.51	734.27	701.01	77.4	64.05	64.24	57.61
5	CAS	685.01	641.22	730.60	624.04	46.97	63.31	67.88	52.85
6	CAS	712.02	754.18	671.82	648.17	46.69	45.19	43.23	39.40
7	CAS	722.01	743.95	645.43	647.07	56.49	45.10	47.81	39.78
8	CAS	656.13	640.05	648.09	656.05	66.20	54.81	49.77	47.25
10	CAS	704.45	705.70	684.31	674.71	71.34	74.23	69.09	59.66
14	CAS	663.86	796.17	668.77	673.69	92.72	87.02	82.07	75.91
16	CAS	627.09	658.31	587.43	593.44	74.51	82.26	78.43	60.13
17	CAS	660.27	675.64	625.06	669.48	46.22	47.06	50.79	47.71
21	CAS	674.32	689.23	642.54	650.90	71.62	69.09	55.28	67.60
	Mean	**681.83**	**703.40**	**663.83**	**653.86**	**65.01**	**63.21**	**60.86**	**54.79**
	S.E.	**9.74**	**16.47**	**14.26**	**9.38**	**4.93**	**4.80**	**4.25**	**3.72**

Table 10. Raw Data for Histidine and Citruline (µM)

Subject	Group	Histidine (µM) d 14 PA	Histidine (µM) d 14 PP	Histidine (µM) d 21 PA	Histidine (µM) d 21 PP	Citruline (µM) d 14 PA	Citruline (µM) d 14 PP	Citruline (µM) d 21 PA	Citruline (µM) d 21 PP
1	WHEY	85.27	98.54	93.95	94.60	43.58	56.55	54.01	55.79
3	WHEY	88.69	77.39	84.54	77.39	42.18	41.79	43.64	41.48
4	WHEY	83.44	87.53	90.3	86.87	48.73	55.15	46.63	52.29
9	WHEY	73.60	78.77	94.6	75.49	45.23	48.98	42.94	40.46
11	WHEY	92.78	90.81	99.93	93.44	43.96	49.62	46.12	54.77
12	WHEY	85.70	89.42	100.00	92.05	44.02	42.94	46.31	45.23
13	WHEY	81.77	77.97	86.21	73.89	53.56	56.62	53.88	57.44
15	WHEY	88.04	79.50	84.61	83.95	49.75	39.69	43.45	40.27
18	WHEY	68.42	60.54	86.51	67.61	52.16	43.58	54.26	39.63
20	WHEY	60.76	68.78	75.42	71.55	34.41	53.82	49.30	57.12
	Mean	**80.85**	**80.93**	**89.61**	**81.68**	**45.76**	**48.87**	**48.05**	**48.45**
	S.E.	**3.19**	**3.52**	**2.43**	**3.10**	**1.76**	**2.06**	**1.43**	**2.43**

Subject	Group	Histidine (µM) d 14 PA	Histidine (µM) d 14 PP	Histidine (µM) d 21 PA	Histidine (µM) d 21 PP	Citruline (µM) d 14 PA	Citruline (µM) d 14 PP	Citruline (µM) d 21 PA	Citruline (µM) d 21 PP
2	CAS	96.72	99.85	96.43	92.63	48.92	55.85	51.78	50.89
5	CAS	83.37	85.92	78.77	79.65	43.70	43.45	52.93	43.83
6	CAS	88.99	97.30	96.79	105.11	54.39	60.05	56.42	60.50
7	CAS	97.81	101.24	101.46	96.64	57.51	61.96	58.97	67.18
8	CAS	94.53	96.86	91.47	89.20	45.23	51.91	45.87	47.33
10	CAS	100.36	102.12	109.70	99.85	32.44	37.28	42.49	43.58
14	CAS	85.12	98.18	90.37	94.89	41.35	53.75	58.59	61.96
16	CAS	82.71	92.78	85.12	95.48	42.43	40.14	50.70	48.85
17	CAS	87.38	93.87	87.75	92.34	49.62	44.97	45.99	50.45
21	CAS	67.98	79.21	52.30	91.25	38.55	43.58	39.25	48.22
	Mean	**88.50**	**94.73**	**89.02**	**93.71**	**45.41**	**49.29**	**50.30**	**52.28**
	S.E.	**3.03**	**2.28**	**4.92**	**2.13**	**2.36**	**2.71**	**2.14**	**2.56**

Table 11. Raw Data for Threonine and Alanine (µM)

Subject	Group	Threonine (µM) d 14 PA	Threonine (µM) d 14 PP	Threonine (µM) d 21 PA	Threonine (µM) d 21 PP	Alanine (µM) d 14 PA	Alanine (µM) d 14 PP	Alanine (µM) d 21 PA	Alanine (µM) d 21 PP
1	WHEY	173.00	245.50	195.05	242.04	346.98	629.25	435.58	719.97
3	WHEY	164.19	168.84	166.17	167.66	423.07	512.52	515.89	620.39
4	WHEY	190.31	228.98	172.40	192.78	548.52	624.89	410.41	592.69
9	WHEY	190.60	220.77	181.31	163.40	447.96	545.43	394.09	438.40
11	WHEY	182.29	238.67	204.75	233.33	523.91	627.00	491.98	691.28
12	WHEY	257.57	277.94	221.86	242.83	511.81	623.07	443.88	621.94
13	WHEY	177.35	214.24	170.82	162.22	465.26	644.73	488.89	557.67
15	WHEY	195.45	234.62	203.76	225.42	615.19	743.88	498.59	752.32
18	WHEY	222.95	255.00	201.88	211.57	593.39	650.77	501.27	599.58
20	WHEY	157.86	198.91	193.57	229.48	382.14	562.31	488.33	685.65
	Mean	**191.16**	**228.35**	**191.16**	**207.07**	**485.82**	**616.39**	**466.89**	**627.99**
	S.E.	**9.35**	**9.59**	**5.69**	**10.40**	**27.93**	**20.38**	**13.40**	**28.73**

Subject	Group	Threonine (µM) d 14 PA	Threonine (µM) d 14 PP	Threonine (µM) d 21 PA	Threonine (µM) d 21 PP	Alanine (µM) d 14 PA	Alanine (µM) d 14 PP	Alanine (µM) d 21 PA	Alanine (µM) d 21 PP
2	CAS	249.46	236.00	256.08	256.08	498.73	577.36	655.41	845.71
5	CAS	144.81	139.86	120.77	114.84	425.32	475.95	362.59	388.61
6	CAS	171.81	173.00	174.48	167.36	443.88	582.56	429.96	602.67
7	CAS	157.57	189.81	180.12	184.77	588.05	671.31	450.21	582.14
8	CAS	213.65	217.21	188.13	209.40	473.70	623.49	446.98	638.40
10	CAS	192.78	233.14	176.46	180.71	595.92	661.18	504.08	611.67
14	CAS	206.13	245.10	242.83	279.92	674.40	829.54	607.88	875.67
16	CAS	165.58	191.49	171.41	178.14	365.40	512.94	468.21	565.96
17	CAS	162.91	196.54	167.95	196.24	406.61	551.90	380.17	556.68
21	CAS	153.51	174.88	139.56	178.73	426.16	547.12	333.05	458.51
	Mean	**181.82**	**199.70**	**181.78**	**194.62**	**489.82**	**603.33**	**463.85**	**612.60**
	S.E.	**10.42**	**10.48**	**12.99**	**14.61**	**31.23**	**31.77**	**32.50**	**47.66**

Table 12. Raw Data for Arginine and Proline (μM)

Subject	Group	Arginine (μM) d 14 PA	Arginine (μM) d 14 PP	Arginine (μM) d 21 PA	Arginine (μM) d 21 PP	Proline (μM) d 14 PA	Proline (μM) d 14 PP	Proline (μM) d 21 PA	Proline (μM) d 21 PP
1	WHEY	52.30	82.33	79.32	87.39	167.77	367.56	185.89	322.04
3	WHEY	52.69	51.28	61.65	56.85	236.25	273.64	233.57	271.06
4	WHEY	87.90	97.18	96.54	92.77	434.91	482.18	225.33	308.34
9	WHEY	116.84	120.61	81.88	68.18	245.73	294.34	186.10	202.06
11	WHEY	82.20	106.02	78.55	88.73	272.91	370.75	236.66	353.76
12	WHEY	68.37	68.31	67.99	67.99	299.79	356.23	213.80	313.90
13	WHEY	98.59	98.34	84.44	70.42	236.97	329.04	180.23	227.81
15	WHEY	89.24	95.39	65.24	64.21	535.32	623.58	319.26	420.91
18	WHEY	103.71	97.76	119.72	103.14	269.10	325.75	177.24	254.99
20	WHEY	77.91	91.55	82.59	90.91	240.37	350.88	197.01	310.09
	Mean	**82.98**	**90.88**	**81.79**	**79.06**	**293.91**	**377.39**	**215.51**	**298.5**
	S.E.	**6.65**	**6.17**	**5.32**	**4.83**	**34.47**	**32.58**	**13.51**	**20.00**

Subject	Group	Arginine (μM) d 14 PA	Arginine (μM) d 14 PP	Arginine (μM) d 21 PA	Arginine (μM) d 21 PP	Proline (μM) d 14 PA	Proline (μM) d 14 PP	Proline (μM) d 21 PA	Proline (μM) d 21 PP
2	CAS	94.43	82.71	100.83	98.08	556.95	349.43	644.49	509.99
5	CAS	111.78	102.56	110.82	102.75	148.20	246.86	133.88	222.97
6	CAS	109.99	136.49	100.45	102.82	265.09	384.24	240.47	367.66
7	CAS	98.85	110.31	87.90	90.65	289.19	400.21	221.32	335.12
8	CAS	68.12	73.50	86.49	91.17	632.34	793.41	389.50	625.54
10	CAS	97.31	95.33	85.72	74.78	388.77	542.12	213.29	305.87
14	CAS	93.09	116.13	94.05	112.23	275.80	434.09	272.81	500.72
16	CAS	73.82	87.13	77.21	87.64	267.77	408.24	196.60	344.90
17	CAS	76.70	95.39	76.89	114.08	321.63	463.03	204.33	444.49
21	CAS	69.78	87.39	75.48	92.96	501.75	620.49	281.36	418.64
	Mean	**89.39**	**98.69**	**89.58**	**96.72**	**364.75**	**464.21**	**279.80**	**407.59**
	S.E.	**5.12**	**5.83**	**3.74**	**3.75**	**48.26**	**48.74**	**45.75**	**37.02**

Table 13. Raw Data for Alpha-aminobutyric Acid and Tyrosine (µM)

Subject	Group	AAB (µM) d 14 PA	AAB (µM) d 14 PP	AAB (µM) d 21 PA	AAB (µM) d 21 PP	Tyrosine (µM) d 14 PA	Tyrosine (µM) d 14 PP	Tyrosine (µM) d 21 PA	Tyrosine (µM) d 21 PP
1	WHEY	39.65	52.24	54.00	53.41	68.20	119.61	83.15	113.97
3	WHEY	31.29	30.00	51.41	40.71	62.44	76.65	81.56	100.18
4	WHEY	26.35	27.18	39.65	37.88	84.93	91.42	66.42	75.74
9	WHEY	26.59	23.18	44.82	34.00	66.30	87.50	66.48	67.65
11	WHEY	28.35	27.06	54.59	49.65	75.55	120.28	79.78	110.85
12	WHEY	37.06	36.94	57.88	52.35	70.83	74.75	67.46	87.87
13	WHEY	25.53	24.24	44.47	33.88	88.60	119.06	86.58	95.22
15	WHEY	19.88	19.88	45.76	41.18	12.01	104.29	75.18	94.61
18	WHEY	32.59	32.12	66.35	55.41	98.47	112.25	87.62	97.55
20	WHEY	22.47	21.88	29.18	30.94	73.65	95.22	83.15	98.90
	Mean	**28.98**	**29.47**	**48.81**	**42.94**	**70.10**	**100.10**	**77.74**	**94.25**
	S.E.	**1.96**	**3.00**	**3.28**	**2.86**	**7.49**	**5.54**	**2.62**	**4.50**

Subject	Group	AAB (µM) d 14 PA	AAB (µM) d 14 PP	AAB (µM) d 21 PA	AAB (µM) d 21 PP	Tyrosine (µM) d 14 PA	Tyrosine (µM) d 14 PP	Tyrosine (µM) d 21 PA	Tyrosine (µM) d 21 PP
2	CAS	31.76	49.76	31.53	49.76	121.26	92.77	127.57	139.46
5	CAS	24.82	22.12	32.94	25.29	61.83	90.99	56.00	76.04
6	CAS	30.12	25.76	37.88	35.06	75.74	94.61	91.79	103.31
7	CAS	31.65	30.94	48.00	46.35	102.63	126.65	95.96	119.49
8	CAS	35.18	28.24	31.88	33.18	84.62	127.45	87.19	128.74
10	CAS	29.18	30.82	34.35	32.12	111.58	153.74	82.41	87.62
14	CAS	28.00	30.71	52.47	50.47	81.31	146.32	106.50	157.66
16	CAS	23.06	24.12	32.47	29.06	71.20	122.24	76.47	117.34
17	CAS	38.71	37.29	62.12	62.35	98.65	130.64	83.58	116.73
21	CAS	27.06	30.71	52.12	62.24	97.30	126.84	74.57	107.17
	Mean	**29.95**	**31.05**	**41.58**	**42.59**	**90.61**	**121.23**	**88.20**	**115.36**
	S.E.	**1.48**	**2.48**	**3.51**	**4.27**	**5.95**	**6.92**	**6.12**	**7.53**

Table 14. Raw Data for Valine and Methionine (μM)

Subject	Group	Valine (μM) d 14 PA	Valine (μM) d 14 PP	Valine (μM) d 21 PA	Valine (μM) D 21 PP	Methionine (μM) d 14 PA	Methionine (μM) d 14 PP	Methionine (μM) d 21 PA	Methionine (μM) d 21 PP
1	WHEY	300.50	536.23	397.07	516.95	31.40	52.67	41.08	53.20
3	WHEY	401.21	455.60	388.50	432.19	31.02	32.62	40.55	37.27
4	WHEY	346.92	417.36	331.28	383.05	33.46	35.75	37.58	34.22
9	WHEY	336.93	409.59	373.66	405.65	30.64	36.51	36.43	28.43
11	WHEY	359.64	552.27	353.18	539.35	33.31	44.44	37.8	46.95
12	WHEY	487.59	511.00	366.09	489.91	35.67	35.21	36.13	41.01
13	WHEY	296.87	370.74	314.13	371.95	36.05	40.55	42.45	34.30
15	WHEY	300.10	348.44	286.38	306.96	37.73	45.20	34.76	39.94
18	WHEY	257.92	306.05	298.99	347.23	45.20	47.10	48.17	48.02
20	WHEY	329.36	450.35	324.32	435.52	28.73	35.59	35.75	40.63
	Mean	**341.71**	**435.76**	**343.36**	**422.86**	**34.32**	**40.56**	**39.07**	**40.40**
	S.E.	**20.50**	**25.74**	**12.03**	**23.76**	**1.49**	**2.06**	**1.29**	**2.34**

Subject	Group	Valine (μM) d 14 PA	Valine (μM) d 14 PP	Valine (μM) d 21 PA	Valine (μM) D 21 PP	Methionine (μM) d 14 PA	Methionine (μM) d 14 PP	Methionine (μM) d 21 PA	Methionine (μM) d 21 PP
2	CAS	360.04	330.58	350.05	376.99	49.16	42.45	50.91	57.39
5	CAS	297.28	385.77	312.31	381.03	32.32	28.13	27.59	30.26
6	CAS	390.41	474.27	403.94	460.95	34.98	46.95	44.28	47.48
7	CAS	363.57	457.82	437.74	522.50	42.61	50.99	43.37	51.14
8	CAS	365.09	450.55	350.86	484.26	38.87	49.31	46.19	53.51
10	CAS	427.55	539.15	340.87	356.21	46.57	58.31	38.26	39.25
14	CAS	279.82	434.61	342.58	443.90	46.72	66.01	51.30	71.65
16	CAS	393.74	521.59	344.10	472.75	25.61	42.00	34.60	42.30
17	CAS	339.46	448.44	424.02	572.15	38.72	54.57	35.14	53.66
21	CAS	448.54	535.02	443.90	575.18	29.12	43.67	34.30	44.36
	Mean	**366.55**	**457.78**	**375.04**	**464.59**	**38.47**	**48.24**	**40.59**	**49.10**
	S.E.	**16.62**	**20.81**	**14.99**	**24.60**	**2.51**	**3.28**	**2.48**	**3.57**

Table 15. Raw Data for Cysteine and Isoleucine (µM)

Subject	Group	Cysteine (µM) d 14 PA	Cysteine (µM) d 14 PP	Cysteine (µM) d 21 PA	Cysteine (µM) d 21 PP	Isoleucine (µM) d 14 PA	Isoleucine (µM) d 14 PP	Isoleucine (µM) d 21 PA	Isoleucine (µM) d 21 PP
1	WHEY	31.86	60.59	49.28	71.90	74.20	192.49	118.37	177.74
3	WHEY	49.61	59.18	70.00	62.16	94.61	128.53	107.86	135.42
4	WHEY	34.59	29.14	35.82	40.20	97.70	157.77	94.96	132.69
9	WHEY	35.82	38.55	34.01	29.88	96.29	141.17	101.50	118.73
11	WHEY	30.29	32.11	29.55	32.11	102.30	216.78	95.32	193.02
12	WHEY	33.18	34.34	39.87	40.61	115.81	146.29	100.27	172.26
13	WHEY	39.13	39.70	32.44	33.18	75.80	107.69	79.51	99.38
15	WHEY	43.09	43.17	40.61	47.22	72.26	108.39	69.08	81.27
18	WHEY	65.87	69.92	75.20	88.32	65.99	87.63	70.14	94.96
20	WHEY	58.44	57.37	72.64	70.00	74.12	139.58	75.18	129.77
	Mean	**42.19**	**46.41**	**47.94**	**51.56**	**89.91**	**142.63**	**91.22**	**133.52**
	S.E.	**3.83**	**4.48**	**5.66**	**6.39**	**5.20**	**12.41**	**5.34**	**11.84**

Subject	Group	Cysteine (µM) d 14 PA	Cysteine (µM) d 14 PP	Cysteine (µM) d 21 PA	Cysteine (µM) d 21 PP	Isoleucine (µM) d 14 PA	Isoleucine (µM) d 14 PP	Isoleucine (µM) d 21 PA	Isoleucine (µM) d 21 PP
2	CAS	62.16	60.59	64.47	82.13	80.74	89.40	81.98	109.81
5	CAS	31.45	34.34	31.86	32.19	86.75	103.09	79.51	106.18
6	CAS	31.53	26.33	39.87	34.09	101.50	133.04	118.37	123.94
7	CAS	37.56	33.76	37.89	43.50	100.27	129.51	110.51	135.42
8	CAS	50.10	48.54	37.39	47.63	122.88	138.60	108.48	151.24
10	CAS	44.33	47.38	46.39	46.80	127.21	167.40	90.11	87.90
14	CAS	41.52	43.75	46.89	48.87	80.21	130.48	84.10	128.18
16	CAS	41.77	43.34	45.56	39.87	100.18	145.67	87.54	131.18
17	CAS	52.99	53.49	55.64	64.47	86.66	117.40	104.33	148.76
21	CAS	72.89	78.01	85.76	110.20	109.54	152.65	121.73	169.52
	Mean	**46.63**	**46.95**	**49.17**	**54.98**	**99.59**	**130.72**	**98.67**	**129.21**
	S.E.	**4.19**	**4.69**	**5.05**	**7.71**	**5.24**	**7.28**	**4.99**	**7.58**

Table 16. Raw Data for Leucine and Phenylalanine (μM)

Subject	Group	Leucine (μM) d 14 PA	Leucine (μM) d 14 PP	Leucine (μM) d 21 PA	Leucine (μM) d 21 PP	Phenylalanine (μM) d 14 PA	Phenylalanine (μM) d 14 PP	Phenylalanine (μM) d 21 PA	Phenylalanine (μM) d 21 PP
1	WHEY	175.88	361.04	244.26	337.81	58.08	72.21	74.93	82.00
3	WHEY	204.15	251.50	225.35	253.36	65.63	59.78	76.83	62.30
4	WHEY	196.02	274.47	193.64	241.78	62.91	57.27	62.50	60.33
9	WHEY	173.23	237.10	210.07	221.64	62.84	60.87	73.57	60.80
11	WHEY	207.60	403.62	215.46	378.09	68.21	71.74	64.27	73.98
12	WHEY	260.95	287.28	227.92	331.89	59.78	63.04	64.67	71.40
13	WHEY	179.33	229.68	195.58	222.70	67.80	63.72	80.03	65.69
15	WHEY	184.19	233.39	183.13	185.34	69.09	65.29	59.58	54.42
18	WHEY	164.93	183.22	180.39	205.65	76.97	66.85	90.35	81.25
20	WHEY	171.11	278.27	182.07	270.85	68.41	70.58	71.40	69.16
	Mean	**191.74**	**273.96**	**205.79**	**264.91**	**64.97**	**65.14**	**71.81**	**68.13**
	S.E.	**8.92**	**20.63**	**7.02**	**20.22**	**1.72**	**1.64**	**2.97**	**2.89**

Subject	Group	Leucine (μM) d 14 PA	Leucine (μM) d 14 PP	Leucine (μM) d 21 PA	Leucine (μM) d 21 PP	Phenylalanine (μM) d 14 PA	Phenylalanine (μM) d 14 PP	Phenylalanine (μM) d 21 PA	Phenylalanine (μM) d 21 PP
2	CAS	190.19	189.58	180.39	221.20	87.57	70.79	77.92	80.30
5	CAS	167.67	197.53	272.17	210.69	60.87	66.78	60.46	65.69
6	CAS	191.78	237.01	220.49	221.11	66.85	71.88	80.37	77.17
7	CAS	195.23	241.34	223.94	265.37	78.46	80.84	80.64	89.13
8	CAS	213.69	240.19	202.74	260.34	72.01	76.90	79.82	86.48
10	CAS	224.56	290.55	191.78	182.95	75.82	85.60	81.11	76.77
14	CAS	179.59	275.97	205.92	276.77	67.12	86.48	80.30	97.69
16	CAS	200.18	287.10	195.67	270.76	65.76	76.77	75.88	81.73
17	CAS	195.23	253.71	238.96	328.62	69.09	77.92	69.90	84.51
21	CAS	236.13	316.34	285.51	375.71	67.26	86.89	73.10	91.03
	Mean	**199.43**	**252.93**	**221.76**	**261.35**	**71.08**	**78.08**	**75.95**	**82.05**
	S.E.	**6.47**	**12.81**	**10.96**	**18.22**	**2.44**	**2.20**	**2.08**	**2.82**

Table 17. Raw Data for Tryptophan and Ornithine (μM)

Subject	Group	Tryptophan (μM) d 14 PA	Tryptophan (μM) d 14 PP	Tryptophan (μM) d 21 PA	Tryptophan (μM) d 21 PP	Ornithine (μM) d 14 PA	Ornithine (μM) d 14 PP	Ornithine (μM) d 21 PA	Ornithine (μM) d 21 PP
1	WHEY	54.56	102.79	59.88	127.28	15.74	31.67	22.64	31.21
3	WHEY	70.35	95.38	76.10	99.57	23.37	27.95	22.91	28.09
4	WHEY	64.34	79.86	62.57	84.00	35.39	41.77	26.03	31.14
9	WHEY	78.25	107.14	58.70	80.24	34.99	40.97	22.51	22.11
11	WHEY	79.97	145.11	76.26	131.53	31.41	38.11	27.36	34.20
12	WHEY	75.99	117.83	54.62	119.55	39.24	40.57	33.73	39.91
13	WHEY	64.34	93.07	66.00	90.01	37.05	45.88	27.09	30.48
15	WHEY	63.86	92.48	55.69	86.41	41.24	47.74	33.20	37.92
18	WHEY	65.84	89.26	66.92	86.41	36.85	52.39	46.41	47.34
20	WHEY	73.90	110.69	74.65	104.94	28.95	39.91	30.88	37.98
	Mean	**69.14**	**103.36**	**65.14**	**100.99**	**32.42**	**40.70**	**29.28**	**34.04**
	S.E.	**2.51**	**5.83**	**2.61**	**6.01**	**2.48**	**2.27**	**2.31**	**2.23**

Subject	Group	Tryptophan (μM) d 14 PA	Tryptophan (μM) d 14 PP	Tryptophan (μM) d 21 PA	Tryptophan (μM) d 21 PP	Ornithine (μM) d 14 PA	Ornithine (μM) d 14 PP	Ornithine (μM) d 21 PA	Ornithine (μM) d 21 PP
2	CAS	81.69	73.20	91.08	108.81	29.55	30.35	35.33	32.74
5	CAS	40.33	56.02	43.66	52.74	31.08	38.84	30.94	39.31
6	CAS	62.57	61.39	75.03	70.68	30.88	42.16	28.29	37.25
7	CAS	89.53	87.33	79.22	102.42	43.36	56.57	37.25	44.95
8	CAS	91.57	95.70	71.64	95.06	64.48	69.52	29.88	38.38
10	CAS	82.76	99.46	73.25	83.89	45.82	53.45	28.75	30.21
14	CAS	73.31	107.36	82.49	111.12	33.00	47.14	33.27	48.41
16	CAS	70.68	79.81	61.49	78.89	31.87	46.15	24.24	39.44
17	CAS	60.31	63.59	49.89	87.22	27.03	40.90	23.24	41.97
21	CAS	75.30	113.37	64.66	112.57	51.33	62.28	42.16	62.08
	Mean	**72.81**	**83.72**	**69.24**	**90.34**	**38.84**	**48.74**	**31.33**	**41.47**
	S.E.	**4.87**	**6.36**	**4.62**	**6.16**	**3.82**	**3.73**	**1.84**	**2.84**

Table 18. Raw Data for Lysine (µM)

Subject	Group	Lysine (µM) d 14 PA	Lysine (µM) d 14 PP	Lysine (µM) d 21 PA	Lysine (µM) d 21 PP
1	WHEY	127.69	268.64	161.00	239.00
3	WHEY	139.78	172.07	150.55	171.29
4	WHEY	148.67	205.54	147.97	186.12
9	WHEY	175.51	227.15	174.10	157.80
11	WHEY	156.71	264.82	174.88	244.31
12	WHEY	190.72	238.38	175.59	232.14
13	WHEY	167.78	221.22	154.45	158.11
15	WHEY	134.56	184.63	121.22	153.74
18	WHEY	190.72	252.03	217.08	243.14
20	WHEY	140.25	202.89	160.06	208.58
	Mean	**157.24**	**223.74**	**163.69**	**199.42**
	S.E.	**7.26**	**10.38**	**7.86**	**12.08**

Subject	Group	Lysine (µM) d 14 PA	Lysine (µM) d 14 PP	Lysine (µM) d 21 PA	Lysine (µM) d 21 PP
2	CAS	169.50	169.34	199.53	215.21
5	CAS	155.69	179.17	131.98	162.71
6	CAS	204.06	283.07	214.20	254.60
7	CAS	204.52	260.06	196.88	223.40
8	CAS	176.83	200.86	191.03	221.22
10	CAS	188.69	228.94	174.96	186.27
14	CAS	156.47	216.38	176.05	257.41
16	CAS	154.76	212.17	158.58	209.59
17	CAS	146.49	233.31	174.02	287.36
21	CAS	127.38	188.14	135.88	203.51
	Mean	**168.44**	**217.15**	**175.31**	**222.13**
	S.E.	**7.97**	**11.28**	**8.52**	**11.51**

Table 19. Raw Data for Percent Weight Change

Subject	Group	% Wt. Change (d 1 - d 21)	% Wt. Change (d 15 - d 21)
1	WHEY	-3.99	-3.85
3	WHEY	-1.72	-3.03
4	WHEY	-3.32	-3.55
9	WHEY	-3.03	-2.88
11	WHEY	-2.34	-2.50
12	WHEY	-4.88	-2.79
13	WHEY	-5.37	-4.34
15	WHEY	-4.04	-0.14
18	WHEY	-3.73	-6.32
20	WHEY	-2.77	-4.31
	Mean	**-3.52**	**-3.37**
	S.E.	**0.35**	**0.50**

Subject	Group	% Wt. Change (d 1 - d 21)	% Wt. Change (d 15 - d 21)
2	CAS	-2.75	-2.50
5	CAS	-5.74	-7.24
6	CAS	-1.37	-3.04
7	CAS	-3.99	-5.62
8	CAS	-2.76	-2.64
10	CAS	0.12	-3.27
14	CAS	-4.00	-4.28
16	CAS	-4.04	-3.16
17	CAS	-1.33	-3.48
21	CAS	-2.65	-4.05
	Mean	**-2.85**	**-3.93**
	S.E.	**0.54**	**0.47**

Table 20. Raw Data for Glutathione

Subject	Group	WBC GSH (µmol/10^6 cells) d 1	WBC GSH (µmol/10^6 cells) d 15	WBC GSH (µmol/10^6 cells) d 21	Whole Blood GSH/g Protein d 1	Whole Blood GSH/mg Protein d 15	Whole Blood GSH/mg Protein d 21
1	WHEY	13.32	16.21	8.90	1460.36	1289.56	1365.36
3	WHEY	6.67	8.81	10.80	1337.90	1414.41	1477.99
4	WHEY	7.17	9.10	13.68	1401.35	1469.26	1365.85
9	WHEY	7.17	8.13	17.30	1312.46	1414.09	1322.84
11	WHEY	4.70	6.62	7.25	1268.61	1315.31	1329.40
12	WHEY	10.83	15.50	12.60	1333.38	1460.43	1376.57
13	WHEY	12.83	7.31	13.40	1119.55	1597.82	1347.29
15	WHEY	6.60	9.13	13.75	1238.58	1369.40	1333.56
18	WHEY	8.70	8.14	9.95	1123.43	1468.25	1208.96
20	WHEY	6.10	7.99	16.35	1450.77	1365.53	1346.41
	Mean	**8.41**	**9.69**	**12.4**	**1316.22**	**1446.35**	**1382.12**
	S.E.	**0.93**	**1.06**	**1.00**	**29.01**	**20.28**	**33.10**

Subject	Group	WBC GSH (µmol/10^6 cells) d 1	WBC GSH (µmol/10^6 cells) d 15	WBC GSH (µmol/10^6 cells) d 21	Whole Blood GSH/g Protein d 1	Whole Blood GSH/mg Protein d 15	Whole Blood GSH/mg Protein d 21
2	CAS	10.22	10.08	10.71	1389.65	1438.13	1537.10
5	CAS	10.37	9.07	16.90	1259.39	1344.91	1358.49
6	CAS	15.60	7.78	7.75	1485.95	1259.04	1457.87
7	CAS	10.40	6.71	5.87	1275.32	1439.14	1482.37
8	CAS	7.31	15.86	15.98	1458.46	1306.47	1365.54
10	CAS	8.74	5.27	12.90	1415.23	2014.95	1506.92
14	CAS	6.07	8.11	16.62	1439.21	1320.88	1346.35
16	CAS	5.06	2.98	9.45	1221.27	1257.36	1362.35
17	CAS	6.37	5.40	9.41	1277.41	1329.62	1281.66
21	CAS	7.46	6.27	9.49	1369.56	1283.39	1139.62
	Mean	**8.76**	**7.75**	**11.51**	**1379.33**	**1393.42**	**1313.86**
	S.E.	**0.97**	**1.11**	**1.23**	**34.71**	**70.79**	**71.08**

Table 21. Raw Data for Cumulative Nitrogen Balance (g)

Subject	Group	Cumulative Nitrogen Balance (g)
1	WHEY	-23.86
3	WHEY	-21.77
4	WHEY	-23.11
9	WHEY	-31.27
11	WHEY	-8.88
12	WHEY	-8.81
13	WHEY	-23.25
15	WHEY	-20.20
18	WHEY	-35.69
20	WHEY	-17.39
	Mean	**-21.42**
	S.E.	**2.68**

Subject	Group	Cumulative Nitrogen Balance (g)
2	CAS	-15.54
5	CAS	-15.45
6	CAS	-14.95
7	CAS	-22.44
8	CAS	-29.48
10	CAS	-22.18
14	CAS	-21.13
16	CAS	-19.19
17	CAS	-19.94
21	CAS	-16.50
	Mean	**-19.68**
	S.E.	**1.41**

Table 22. Raw Data for Urine Volume (mL)

Subject	Group	Urine Volume (mL) d 18	Urine Volume (mL) d 19	Urine Volume (mL) d 20	Urine Volume (mL) d 21
1	WHEY	3390	3990	2200	1070
3	WHEY	3330	3315	2720	3015
4	WHEY	4260	2980	3620	2315
9	WHEY	2675	3360	4060	3990
11	WHEY	4200	3970	1540	2680
12	WHEY	2930	3100	2360	2600
13	WHEY	5120	4850	5100	4500
15	WHEY	9330	9560	9940	10050
18	WHEY	3680	3300	3500	3300
20	WHEY	5710	6520	3970	4615
	Mean	**4462.5**	**4494.5**	**3901.0**	**3813.5**
	S.E.	**618.1**	**656.3**	**748.4**	**770.8**

Subject	Group	Urine Volume (mL) d 18	Urine Volume (mL) d 19	Urine Volume (mL) d 20	Urine Volume (mL) d 21
2	CAS	4860	3940	4790	3430
5	CAS	3300	1330	2260	2610
6	CAS	2240	970	1870	2000
7	CAS	3750	2040	3520	3470
8	CAS	7320	7570	7860	7820
10	CAS	2990	4590	2880	4940
14	CAS	3590	1830	2220	2520
16	CAS	4480	3380	2790	2610
17	CAS	4680	4030	4640	5140
21	CAS	4300	3960	3740	4130
	Mean	**4151.0**	**3364.0**	**3657.0**	**3867.0**
	S.E.	**436.6**	**617.3**	**562.8**	**550.5**

Table 23. Raw Data for Urinary Nitrogen Concentration (mg/L)

Subject	Group	Urinary N (mg/L) d 18	Urinary N (mg/L) d 19	Urinary N (mg/L) d 20	Urinary N (mg/L) d 21
1	WHEY	5074.00	4976.45	7460.50	16364.95
3	WHEY	5069.95	6808.40	6740.85	5957.80
4	WHEY	5420.75	6126.40	5042.35	8388.55
9	WHEY	8064.90	5436.50	4488.9	5252.35
11	WHEY	3558.90	3385.85	7195.60	5128.00
12	WHEY	5003.45	4313.25	6145.80	6174.50
13	WHEY	3579.75	3373.50	3631.70	3709.25
15	WHEY	2042.25	1652.85	1687.70	1976.90
18	WHEY	5297.65	6555.40	6726.95	7413.60
20	WHEY	3225.90	2690.05	3371.25	3685.35
	Mean	**4633.75**	**5431.87**	**5249.16**	**5405.13**
	S.E.	**519.81**	**549.95**	**608.63**	**1254.49**

Subject	Group	Urinary N (mg/L) d 18	Urinary N (mg/L) d 19	Urinary N (mg/L) d 20	Urinary N (mg/L) d 21
2	CAS	3416.75	3844.90	3817.55	4913.90
5	CAS	4521.30	11544.75	7335.55	6308.30
6	CAS	7544.30	14171.95	7811.35	7078.25
7	CAS	5357.45	8550.80	4860.00	5480.95
8	CAS	3284.10	2582.95	2224.15	2627.00
10	CAS	6990.00	4419.70	5876.25	3954.55
14	CAS	5579.85	7820.80	7532.50	7119.65
16	CAS	4424.05	4857.75	5951.90	6827.70
17	CAS	4130.70	4319.40	3598.05	2881.75
21	CAS	4179.80	4218.40	4166.30	4356.45
	Mean	**4942.83**	**6633.14**	**5317.36**	**5154.85**
	S.E.	**450.99**	**1199.35**	**598.35**	**532.17**

Appendix C: Statistical Analyses of Measurements

Statistical Procedures

Baseline characteristics and cumulative NBAL of the two groups were compared using independent samples t-tests and paired samples t-tests, respectively. Nitrogen balance, performance and GSH data were analyzed using the repeated measures ANOVA function within the SPSS statistical package with group and day as main factors. Plasma AA were analyzed using repeated measures ANOVA with a 2x2x2 factorial design with group (WHEY and CAS), day (d 14 and d 21) and state (postabsorptive and postprandial) as main factors. Post-hoc pairwise t-tests, with Bonferroni correction for multiple comparisons where appropriate, were performed on analyses with significant omnibus F in ANOVA analyses. Correlations between AA concentrations and total NBAL are represented by Pearson's correlation coefficients and were arrived at using bivariate correlation analyses with a two-tailed test of significance. A p value of less than or equal to 0.05 was determined *a priori* as the standard for significance.

Table 24. RMANOVA for Glutamic Acid

Source	SS	DF	MS	F	P
GROUP	95.936	1	95.936	0.350	0.562
Error	4935.246	18	274.180		
DAY	335.489	1	335.489	10.341	0.005
GROUP * DAY	0.958	1	0.958	0.030	0.866
Error(DAY)	583.980	18	32.443		
STATE	265.924	1	265.924	6.915	0.017
GROUP * STATE	19.279	1	19.279	0.501	0.488
Error(STATE)	692.163	18	38.454		
DAY * STATE	17.788	1	17.788	0.892	0.357
GROUP * DAY * STATE	31.785	1	31.785	1.594	0.223
Error(DAY*STATE)	358.843	18	19.936		

Table 25. RMANOVA for Serine

Source	SS	DF	MS	F	P
GROUP	4302.447	1	4302.447	5.784	0.027
Error	13389.531	18	743.863		
DAY	611.204	1	611.204	2.983	0.101
GROUP * DAY	99.732	1	99.732	0.487	0.494
Error(DAY)	3687.508	18	204.862		
STATE	1805.218	1	1805.218	12.803	0.002
GROUP * STATE	429.221	1	429.221	3.044	0.098
Error(STATE)	2537.976	18	140.999		
DAY * STATE	333.175	1	333.175	11.415	0.003
GROUP * DAY * STATE	187.235	1	187.235	6.415	0.021
				GROUP @ D14 @ PA	0.053
				GROUP @ D14 @ PP	0.031
				GROUP @ D21 @ PA	0.487
				GROUP @ D21 @ PP	0.084
				WHEY @ STATE Δ @ DAY	0.003
				CAS @ STATE Δ @ DAY	0.551
				GROUP @ STATE Δ @ DAY 14	0.544
				GROUP @ STATE Δ @ DAY 21	0.045
Error(DAY*STATE)	525.393	18	29.189		

Table 26. RMANOVA for Asparagine

Source	SS	DF	MS	F	P
GROUP	913.043	1	913.043	0.973	0.337
Error	16886.018	18	938.112		
DAY	47.039	1	47.039	0.391	0.540
GROUP * DAY	7.102	1	7.102	0.059	0.811
Error(DAY)	2164.858	18	120.270		
STATE	3414.585	1	3414.585	18.165	< 0.001
GROUP *STATE	77.069	1	77.069	0.410	0.530
Error(STATE)	3383.661	18	187.981		
DAY * STATE	104.632	1	104.632	6.101	0.024
GROUP * DAY * STATE	268.371	1	268.371	15.647	0.001
				GROUP @ D14 @ PA	0.419
				GROUP @ D14 @ PP	0.570
				GROUP @ D21 @ PA	0.816
				GROUP @ D21 @ PP	0.416
				WHEY @ STATE Δ @ DAY	0.003
				CAS @ STATE Δ @ DAY	0.270
				GROUP @ STATE Δ @ DAY 14	0.672
				GROUP @ STATE Δ @ DAY 21	0.159
Error(DAY*STATE)	308.722	18	17.151		

Table 27. RMANOVA for Glycine

Source	SS	DF	MS	F	P
GROUP	4809.887	1	4809.887	0.421	0.524
Error	205423.839	18	11412.436		
DAY	1001.199	1	1001.199	0.747	0.399
GROUP *DAY	417.868	1	417.868	0.312	0.583
Error(DAY)	24127.367	18	1340.409		
STATE	4799.030	1	4799.030	5.623	0.029
GROUP * STATE	1604.904	1	1604.904	1.881	0.187
Error(STATE)	15361.688	18	853.427		
DAY * STATE	157.035	1	157.035	0.572	0.459
GROUP * DAY * STATE	318.880	1	318.880	1.161	0.296
Error(DAY*STATE)	4944.798	18	274.711		

Table 28. RMANOVA for Glutamine

Source	SS	DF	MS	F	P
GROUP	2844.699	1	2844.699	0.314	0.582
Error	163128.880	18	9062.715		
DAY	11143.548	1	11143.548	5.578	0.030
GROUP * DAY	2066.905	1	2066.905	1.035	0.323
Error(DAY)	35960.534	18	1997.807		
STATE	1977.426	1	1977.426	0.930	0.348
GROUP * STATE	4952.279	1	4952.279	2.330	0.144
Error(STATE)	38261.347	18	2125.630		
DAY * STATE	6829.989	1	6829.989	12.464	0.002
				DAY @ PA	0.650
				DAY @ PP	0.002
				STATE Δ @ DAY	0.002
GROUP * DAY * STATE	146.966	1	146.966	0.268	0.611
Error(DAY*STATE)	9863.880	18	547.993		

Table 29. RMANOVA for Taurine

Source	SS	DF	MS	F	P
GROUP	1.957	1	1.957	0.002	0.966
Error	18930.516	18	1051.695		
DAY	189.305	1	189.305	4.314	0.052
GROUP * DAY	206.333	1	206.333	4.702	0.044
				GROUP @ D14	0.639
				GROUP @ D21	0.496
				DAY Δ @ GROUP	0.042
Error(DAY)	789.926	18	43.885		
STATE	292.388	1	292.388	8.310	0.010
GROUP * STATE	0.251	1	0.251	0.007	0.934
Error(STATE)	633.335	18	35.185		
DAY * STATE	57.756	1	57.756	0.929	0.348
GROUP * DAY * STATE	3.770	1	3.770	0.061	0.808
Error(DAY*STATE)	1118.729	18	62.152		

Table 30. RMANOVA for Histidine

Source	SS	DF	MS	F	P
GROUP	1352.059	1	1352.059	4.397	0.050
Error	5535.065	18	307.504		
DAY	101.431	1	101.431	5.098	0.037
GROUP * DAY	125.730	1	125.730	6.319	0.022
				GROUP @ D14	< 0.001
				GROUP @ D21	0.043
				DAY Δ @ GROUP	0.028
Error(DAY)	358.154	18	19.897		
STATE	11.899	1	11.899	0.225	0.641
GROUP * STATE	440.266	1	440.266	8.316	0.010
				GROUP @ PA	0.145
				GROUP @ PP	< 0.001
				STATE Δ @ GROUP	0.002
Error(STATE)	952.908	18	52.939		
DAY * STATE	113.950	1	113.95	4.478	0.049
				DAY @ PA	0.028
				DAY @ PP	0.911
				STATE Δ @ DAY	0.053
GROUP * DAY * STATE	52.086	1	52.086	2.047	0.170
Error(DAY*STATE)	458.015	18	25.445		

Table 31. RMANOVA for Citruline

Source	SS	DF	MS	F	P
GROUP	47.300	1	47.300	0.358	0.557
Error	2377.796	18	132.100		
DAY	118.565	1	118.565	5.998	0.025
GROUP * DAY	44.981	1	44.981	2.275	0.149
Error(DAY)	355.838	18	19.769		
STATE	109.751	1	109.751	3.091	0.096
GROUP * STATE	6.887	1	6.887	0.194	0.665
Error(STATE)	639.081	18	35.504		
DAY * STATE	26.735	1	26.735	2.908	0.105
GROUP * DAY * STATE	0.842	1	0.842	0.092	0.766
Error(DAY*STATE)	165.510	18	9.195		

Table 32. RMANOVA for Threonine

Source	SS	DF	MS	F	P
GROUP	4471.854	1	4471.854	1.188	0.290
Error	67729.296	18	3762.739		
DAY	871.174	1	871.174	1.803	0.196
GROUP * DAY	326.122	1	326.122	0.675	0.422
Error(DAY)	8696.296	18	483.128		
STATE	8783.895	1	8783.895	31.534	< 0.001
GROUP * STATE	626.292	1	626.292	2.248	0.151
Error(STATE)	5013.974	18	278.554		
DAY * STATE	865.959	1	865.959	11.238	0.004
				DAY @ PA	0.997
				DAY @ PP	0.038
				STATE Δ @ DAY	0.006
GROUP * DAY * STATE	329.325	1	329.325	4.274	0.053
Error(DAY*STATE)	1387.059	18	77.059		

Table 33. RMANOVA for Alanine

Source	SS	DF	MS	F	P
GROUP	944.104	1	944.104	0.037	0.850
Error	463354.600	18	25741.922		
DAY	721.351	1	721.351	0.094	0.763
GROUP * DAY	109.678	1	109.678	0.014	0.906
Error(DAY)	137958.43	18	7664.357		
STATE	383539.820	1	383539.820	121.077	< 0.001
GROUP * STATE	1080.099	1	1080.099	0.341	0.567
Error(STATE)	57019.116	18	3167.729		
DAY * STATE	5406.545	1	5406.545	5.376	0.032
				DAY @ PA	0.271
				DAY @ PP	0.620
				STATE Δ @ DAY	0.028
GROUP * DAY * STATE	27.584	1	27.584	0.027	0.870
Error(DAY*STATE)	18101.421	18	1005.635		

Table 34. RMANOVA for Arginine

Source	SS	DF	MS	F	P
GROUP	1967.483	1	1967.483	2.836	0.109
Error	12486.047	18	693.669		
DAY	273.147	1	273.147	1.034	0.323
GROUP * DAY	157.439	1	157.439	0.596	0.450
Error(DAY)	4754.216	18	264.123		
STATE	583.573	1	583.573	4.444	0.049
GROUP * STATE	158.879	1	158.879	1.210	0.286
Error(STATE)	2363.712	18	131.317		
DAY * STATE	205.136	1	205.136	9.500	0.006
				DAY @ PA	0.880
				DAY @ PP	0.120
				STATE Δ @ DAY	0.010
GROUP * DAY * STATE	89.403	1	89.403	4.140	0.057
Error(DAY*STATE)	388.684	18	21.594		

Table 35. RMANOVA for Proline

Source	SS	DF	MS	F	P
GROUP	136984.488	1	136984.488	3.466	0.079
Error	711465.300	18	39525.850		
DAY	111651.965	1	111651.965	13.058	0.002
GROUP * DAY	309.541	1	309.541	0.036	0.851
Error(DAY)	153907.368	18	8550.409		
STATE	193767.149	1	193767.149	29.957	< 0.001
GROUP * STATE	4618.171	1	4618.171	0.714	0.409
Error(STATE)	116425.911	18	6468.106		
DAY * STATE	967.926	1	967.926	2.107	0.164
GROUP * DAY * STATE	1037.927	1	1037.927	2.260	0.150
Error(DAY*STATE)	8268.419	18	459.357		

Table 36. RMANOVA for Alpha-aminobutyric Acid

Source	SS	DF	MS	F	P
GROUP	31.693	1	31.693	0.132	0.721
Error	4319.720	18	239.984		
DAY	3986.159	1	3986.159	51.404	< 0.001
GROUP * DAY	128.554	1	128.554	1.658	0.214
Error(DAY)	1395.819	18	77.546		
STATE	13.371	1	13.371	0.432	0.519
GROUP * STATE	69.982	1	69.982	2.260	0.150
Error(STATE)	557.270	18	30.959		
DAY * STATE	51.956	1	51.956	9.814	0.006
GROUP * DAY * STATE	49.335	1	49.335	9.319	0.007
				GROUP @ D14 @ PA	0.615
				GROUP @ D14 @ PP	0.417
				GROUP @ D21 @ PA	0.177
				GROUP @ D21 @ PP	0.948
				WHEY @ STATE Δ @ DAY	0.002
				CAS @ STATE Δ @ DAY	0.958
				GROUP @ STATE Δ @ DAY 14	0.635
				GROUP @ STATE Δ @ DAY 21	0.004
Error(DAY*STATE)	95.291	18	5.294		

Table 37. RMANOVA for Tyrosine

Source	SS	DF	MS	F	P
GROUP	6698.652	1	6698.652	9.651	0.006
Error	12494.228	18	694.124		
DAY	52.633	1	52.633	0.156	0.698
GROUP * DAY	126.691	1	126.691	0.375	0.548
Error(DAY)	6083.324	18	337.962		
STATE	13593.703	1	13593.703	46.226	< 0.001
GROUP * STATE	158.031	1	158.031	0.537	0.473
Error(STATE)	5293.235	18	294.069		
DAY * STATE	359.325	1	359.325	2.993	0.101
GROUP * DAY * STATE	125.767	1	125.767	1.048	0.320
Error(DAY*STATE)	2161.083	18	120.060		

Table 38. RMANOVA for Valine

Source	SS	DF	MS	F	P
GROUP	18075.761	1	18075.761	1.646	0.216
Error	197615.819	18	10978.657		
DAY	20.672	1	20.672	0.008	0.931
GROUP * DAY	879.719	1	879.719	0.330	0.573
Error(DAY)	47955.165	18	2664.176		
STATE	156963.083	1	156963.083	66.538	< 0.001
GROUP * STATE	65.069	1	65.069	0.028	0.870
Error(STATE)	42461.679	18	2358.982		
DAY * STATE	328.696	1	328.696	0.504	0.487
GROUP * DAY * STATE	206.911	1	206.911	0.317	0.580
Error(DAY*STATE)	11747.201	18	652.622		

Table 39. RMANOVA for Methionine

Source	SS	DF	MS	F	P
GROUP	607.770	1	607.770	3.553	0.076
Error	3079.032	18	171.057		
DAY	71.605	1	71.605	2.796	0.112
GROUP * DAY	3.172	1	3.172	0.124	0.729
Error(DAY)	460.943	18	25.608		
STATE	835.022	1	835.022	20.992	< 0.001
GROUP * STATE	143.349	1	143.349	3.604	0.074
Error(STATE)	716.019	18	39.779		
DAY * STATE	47.762	1	47.762	4.260	0.054
GROUP * DAY * STATE	16.661	1	16.661	1.486	0.239
Error(DAY*STATE)	201.809	18	11.212		

Table 40. RMANOVA for Cysteine

Source	SS	DF	MS	F	P
GROUP	115.994	1	115.994	0.113	0.741
Error	18487.860	18	1027.103		
DAY	576.199	1	576.199	9.003	0.008
GROUP * DAY	0.143	1	0.143	0.002	0.963
Error(DAY)	1152.018	18	64.001		
STATE	243.545	1	243.545	5.001	0.038
GROUP * STATE	3.649	1	3.649	0.075	0.787
Error(STATE)	875.670	18	48.704		
DAY * STATE	29.749	1	29.749	1.513	0.234
GROUP * DAY * STATE	46.262	1	46.262	2.353	0.142
Error(DAY*STATE)	353.825	18	19.657		

Table 41. RMANOVA for Isoleucine

Source	SS	DF	MS	F	P
GROUP	19.144	1	19.144	0.012	0.915
Error	29313.970	18	1628.554		
DAY	65.431	1	65.431	0.208	0.654
GROUP * DAY	6.954	1	6.954	0.022	0.883
Error(DAY)	5662.077	18	314.560		
STATE	31883.498	1	31883.498	65.880	< 0.001
GROUP * STATE	1651.798	1	1651.798	3.413	0.081
Error(STATE)	8711.374	18	483.965		
DAY * STATE	245.062	1	245.062	1.806	0.196
GROUP * DAY * STATE	205.944	1	205.944	1.518	0.234
Error(DAY*STATE)	2442.726	18	135.707		

Table 42. RMANOVA for Leucine

Source	SS	DF	MS	F	P
GROUP	1.075	1	1.075	0.000	0.988
Error	85919.950	18	4773.331		
DAY	1597.657	1	1597.657	1.824	0.194
GROUP * DAY	828.885	1	828.885	0.946	0.344
Error(DAY)	15767.547	18	875.975		
STATE	68704.672	1	68704.672	35.062	< 0.001
GROUP * STATE	2909.119	1	2909.119	1.485	0.239
Error(STATE)	35271.223	18	1959.512		
DAY * STATE	1711.740	1	1711.740	3.430	0.080
GROUP * DAY * STATE	105.305	1	105.305	0.211	0.651
Error(DAY*STATE)	8983.020	18	499.057		

Table 43. RMANOVA for Phenylalanine

Source	SS	DF	MS	F	P
GROUP	1721.697	1	1721.697	14.43	0.001
Error	2147.624	18	119.312		
DAY	435.957	1	435.957	12.087	0.003
GROUP * DAY	1.247	1	1.247	0.035	0.855
Error(DAY)	649.207	18	36.067		
STATE	114.855	1	114.855	1.957	0.179
GROUP * STATE	433.424	1	433.424	7.386	0.014
				GROUP @ PA	0.112
				GROUP @ PP	< 0.001
				STATE Δ @ GROUP	0.010
Error(STATE)	1056.329	18	58.685		
DAY * STATE	9.462	1	9.462	0.623	0.440
GROUP * DAY * STATE	10.816	1	10.816	0.713	0.410
Error(DAY*STATE)	273.229	18	15.179		

Table 44. RMANOVA for Tryptophan

Source	SS	DF	MS	F	P
GROUP	634.475	1	634.475	0.946	0.344
Error	12068.077	18	670.449		
DAY	13.725	1	13.725	0.128	0.725
GROUP * DAY	111.047	1	111.047	1.036	0.322
Error(DAY)	1929.550	18	107.197		
STATE	13027.744	1	13027.744	61.845	< 0.001
GROUP * STATE	1810.821	1	1810.821	8.596	0.009
				GROUP @ PA	0.111
				GROUP @ PP	0.518
				STATE Δ @ GROUP	0.002
Error(STATE)	3791.751	18	210.653		
DAY * STATE	174.342	1	174.342	3.850	0.065
GROUP * DAY * STATE	91.262	1	91.262	2.016	0.173
Error(DAY*STATE)	815.032	18	45.280		

Table 45. RMANOVA for Ornithine

Source	SS	DF	MS	F	P
GROUP	717.055	1	717.055	3.614	0.073
Error	3571.337	18	198.408		
DAY	754.915	1	754.915	8.982	0.008
GROUP * DAY	30.754	1	30.754	0.366	0.553
Error(DAY)	1512.889	18	84.049		
STATE	1367.392	1	1367.392	71.520	0.000
GROUP * STATE	61.343	1	61.343	3.208	0.090
Error(STATE)	344.143	18	19.119		
DAY * STATE	13.395	1	13.395	2.092	0.165
GROUP * DAY * STATE	17.594	1	17.594	2.748	0.115
Error(DAY*STATE)	115.231	18	6.402		

Table 46. RMANOVA for Lysine

Source	SS	DF	MS	F	P
GROUP	1895.322	1	1895.322	0.784	0.388
Error	43503.621	18	2416.868		
DAY	45.094	1	45.094	0.093	0.764
GROUP * DAY	1104.038	1	1104.038	2.265	0.150
Error(DAY)	8773.593	18	487.422		
STATE	48883.063	1	48883.063	63.127	< 0.001
GROUP * STATE	56.251	1	56.251	0.073	0.791
Error(STATE)	13938.553	18	774.364		
DAY * STATE	1332.700	1	1332.700	9.474	0.006
GROUP * DAY * STATE	1042.334	1	1042.334	7.410	0.014
				GROUP @ D14 @ PA	0.257
				GROUP @ D14 @ PP	0.684
				GROUP @ D21 @ PA	0.298
				GROUP @ D21 @ PP	0.181
				WHEY @ STATE Δ @ DAY	0.003
				CAS @ STATE Δ @ DAY	0.800
				GROUP @ STATE Δ @ DAY 14	0.315
				GROUP @ STATE Δ @ DAY 21	0.501
Error(DAY*STATE)	2532.077	18	140.671		

Table 47. RMANOVA for Branched Chain Amino Acids

Source	SS	DF	MS	F	P
GROUP	18984.548	1	18984.548	0.444	0.513
Error	768967.708	18	42720.428		
DAY	1327.024	1	1327.024	0.154	0.699
GROUP * DAY	3731.680	1	3731.680	0.434	0.518
Error(DAY)	154759.793	18	8597.766		
STATE	700336.227	1		56.476	< 0.001
GROUP * STATE	7484.330	1	7484.330	0.604	0.447
Error(STATE)	223211.373	18	12400.632		
DAY * STATE	5648.666	1	5648.666	1.715	0.207
GROUP * DAY * STATE	1520.762	1	1520.762	0.462	0.506
Error(DAY*STATE)	59296.247	18	3294.236		

Table 48. RMANOVA for Essential Amino Acids

Source	SS	DF	MS	F	P
GROUP	36931.498	1	36931.498	0.524	0.479
Error	1269746.957	18	70541.498		
DAY	1289.209	1	1289.209	0.082	0.778
GROUP * DAY	12328.502	1	12328.502	0.784	0.388
Error(DAY)	283176.041	18	15732.002		
STATE	1713169.323	1	1713169.323	54.477	< 0.001
GROUP * STATE	11624.610	1	11624.610	0.370	0.551
Error(STATE)	566056.140	18	31447.563		
DAY * STATE	22066.799	1	22066.799	3.306	0.086
GROUP * DAY * STATE	12898.208	1	12898.208	1.932	0.181
Error(DAY*STATE)	120152.937	18	6675.163		

Table 49. RMANOVA for Nonessential Amino Acids

Source	SS	DF	MS	F	P
GROUP	321650.263	1	321650.263	1.534	0.231
Error	3773640.192	18	209646.677		
DAY	221966.895	1	221966.895	3.731	0.069
GROUP * DAY	1768.804	1	1768.804	0.030	0.865
Error(DAY)	1070933.566	18	59496.309		
STATE	1480868.577	1	1480868.577	32.052	< 0.001
GROUP * STATE	36268.595	1	36268.595	0.785	0.387
Error(STATE)	831626.924	18	46201.496		
DAY * STATE	1625.422	1	1625.422	0.219	0.645
GROUP * DAY * STATE	21065.859	1	21065.859	2.839	0.109
Error(DAY*STATE)	133558.197	18	7419.900		

Table 50. RMANOVA for Sulfur Amino Acids

Source	SS	DF	MS	F	P
GROUP	1254.845	1	1254.845	0.927	0.349
Error	24377.041	18	1354.280		
DAY	1054.007	1	1054.007	8.886	0.008
GROUP * DAY	4.685	1	4.685	0.039	0.845
Error(DAY)	2135.108	18	118.617		
STATE	1980.846	1	1980.846	15.775	0.001
GROUP * STATE	101.205	1	101.205	0.806	0.381
Error(STATE)	2260.291	18	125.572		
DAY * STATE	2.126	1	2.126	0.050	0.825
GROUP * DAY * STATE	118.438	1	118.438	2.804	0.111
Error(DAY*STATE)	760.262	18	42.237		

Table 51. RMANOVA for Time-to-Exhasution

Source	SS	DF	MS	F	P
GROUP	458.111	1	458.111	0.037	0.851
Error	174622.196	14	12473.014		
DAY	29102.460	2	14551.230	2.763	0.073
GROUP * DAY	29999.128	2	14999.564	2.848	0.072
Error(DAY)	147448.504	28	5266.018		

Table 52. RMANOVA for Body Weight

Source	SS	DF	MS	F	P
GROUP	32.708	1	32.708	0.157	0.697
Error	3761.028	18	208.946		
DAY	83.449	2	41.725	60.116	< 0.001
GROUP * DAY	1.937	2	0.969	1.396	0.261
Error(DAY)	24.987	36	0.694		

Table 53. RMANOVA for Nitrogen Balance

Source	SS	DF	MS	F	P
GROUP	3.792	1	3.792	0.330	0.573
Error	206.731	18	11.485		
DAY	46.510	3	15.503	6.250	0.001
				DAY 18 - DAY 19	0.013
				DAY 18 - DAY 20	0.002
				DAY 18 - DAY 21	0.070
				DAY 19 - DAY 20	0.457
				DAY 19 - DAY 21	0.135
				DAY 20 - DAY 21	0.007
GROUP * DAY	8.233	3	2.744	1.106	0.355
Error(DAY)	133.944	54	2.480		

Table 54. RMANOVA for Whole Blood GSH

Source	SS	DF	MS	F	P
GROUP	5621.757	1	5621.757	0.145	0.708
Error	698756.440	18	38819.802		
DAY	69124.311	2	34562.155	2.272	0.133
GROUP * DAY	51602.257	2	25801.128	2.071	0.157
Error(DAY)	526703.230	36	14630.645		

Table 55. RMANOVA for WBC GSH

Source	SS	DF	MS	F	P
GROUP	10.251	1	10.251	0.738	0.401
Error	249.875	18	13.882		
DAY	145.305	2	72.653	5.356	0.016
GROUP * DAY	13.163	2	6.582	1.008	0.386
Error(DAY)	351.955	36	9.777		

Table 56. Independent Samples t-tests for Baseline Characteristics

	T	DF	P
AGE	1.380	18	0.184
HT	0.885	18	0.388
V02PEAK	-1.350	18	0.194
BODYFAT	0.854	18	0.405

Table 57. Paired Samples t-tests for 4 d Cumulative Nitrogen Balance

	T	DF	P
TOTAL NBAL	-0.503	9	0.627

Appendix D: Health History Questionnaire

VIRGINIA TECH LABORATORY FOR HEALTH AND EXERCISE SCIENCE
MEDICAL AND HEALTH HISTORY

Name: _____ Age: _____ Birth Date: _____

Address: _____ e-mail: _____

Phone Numbers: Home: _____ Work :_____

Summer Address:

_____

_____

Phone Number (during Winter Break): _____

Person to Contact in Case of an Emergency:

_____

Relationship: _____Phone: _____

Primary Care Physician: _____Phone: _____

Medical Insurance Carrier: _____

Are you employed by Virginia Tech? _____

Current Body Weight:_____

MEDICAL HISTORY

Please indicate any current or previous conditions or problems you have experienced or have been told by a physician you have had:

	Yes	**No**
Heart disease or any heart problems:	_____	_____
Rheumatic Fever:	_____	_____
Respiratory disease or breathing problems (e.g. asthma):	_____	_____
Circulation problems:	_____	_____
Kidney disease or problems:	_____	_____
Urinary problems:	_____	_____
Musculoskeletal problems:	_____	_____

(i.e. Orthopedic injuries, osteoporosis)

Fainting and Dizziness:	_____	_____
High Cholesterol:	_____	_____
Diabetes:	_____	_____
Thyroid problems:	_____	_____
Mental illness:	_____	_____
Hypoglycemia:(i.e. low blood sugar)	_____	_____
Epilepsy or seizures:	_____	_____
Blood clotting problems (e.g. hemophilia):	_____	_____
Liver disorders (e.g. hepatitis B*)*	_____	_____

If you answered "yes" to any of the previous questions, please indicate the date and describe:

_____
_____
_____
_____

Please list any hospitalizations/operations/recent illnesses (type/date):

_____
_____
_____
_____
_____

	Yes	**No**
Have you ever been diagnosed as having high blood pressure?	_____	_____
Are you currently being treated for high blood pressure?	_____	_____

If "yes", please explain:

_____
_____
_____

Please list all medications (prescription and over-the-counter) you are currently taking or have taken in the past week:

_____
_____
_____

For what reason(s) are you taking this medication?

_____

_____
_____

Health Habits

	Yes	**No**
Do you drink alcoholic beverages?	_____	_____
How many drinks per week? _____		
Do you smoke cigarettes?	_____	_____
Packs per day: _____		

	Yes	**No**
Do you engage in regular exercise?	_____	_____

If "yes", please list:

Activity	**Frequency (times per week)**	**Duration (minutes)**
_____	_____	_____
_____	_____	_____
_____	_____	_____

Do you ever faint, experience shortness of breath or chest discomfort with exertion?
_____

If "yes", please explain: _____
_____

Are there any orthopedic limitations you have that may restrict your ability to perform exercise and if "yes", please explain:
_____
_____
_____

Family History

Has anyone in your family been diagnosed or treated for any of the following?

	Yes	**No**	**Relationship**	**Age**
Heart attack	____	____	_____	____
Heart disease	____	____	_____	____
High blood pressure	____	____	_____	____
Stroke	____	____	_____	____
Kidney disease	____	____	_____	____
Diabetes	____	____	_____	____

Schedule Spring 2001 semester (indicate those times you have classes, work etc that you CANNOT be involved in testing or exercise training):

	Mon	*Tue*	*Wed*	*Thursday*	*Fri*
6:00-7:00am					
7:00-8:00					
8:00-9:00					
9:00-10:00					
10:00-11:00					
11:00-12:00					
12:00-1:00					
1:00-2:00					
2:00-3:00					
3:00-4:00					
4:00-5:00					
5:00-6:00					
6:00-7:00					
7:00-8:00					

Any explanation required for above

_____

_____

_____

Please sign to indicate that the above information is correct:

_____	_____	_____
Print name	Signature	Date

SUPPLEMENTARY QUESTIONS

Food Habits and Allergies

1. Are you allergic to any foods? ____ If yes, which ones?

2. Are you on any kind of special diet? ____ If so, what kind?

1. Do you take any dietary supplements?_____ If so, what kind and how often?

2. Has your weight been stable over the past year?____ If not, how has it changed?

Drug Allergies

3. Are you aware of any allergies you have to any drugs? ____ If yes, which ones?

4. Have you ever received Novocaine at the dentist's office or other local (injected into skin) anesthetic? _____.

If yes, did you have any allergic reaction to this?_____

Comfort with procedures

5. Do you have a fear of needles or having blood withdrawn

Appendix E: Institutional Review Board Request

Request for Approval of Research Proposal
Department of Human Nutrition, Foods, and Exercise
Virginia Tech

TITLE: Value of Whey Protein Isolate on Glutathione Status, Exercise Performance, and Immunity in Men During Energy Balance and Restriction

FACULTY INVESTIGATORS:

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STUDENT INVESTIGATORS:

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Masters Graduate student
Department of Human Nutrition, Foods, and Exercise

I. Literature Review and Justification of Project

Free radicals are produced during exercise and may be associated with fatigue.

The elevated use of oxygen that occurs during aerobic exercise has been shown to cause an increase in production of reactive oxygen species (ROS, e.g. hydrogen peroxide, superoxide) within the active muscle (McArdle, 2001). These compounds can be managed by antioxidants within the body such as glutathione, ascorbate, etc. However, production of ROS at a level beyond the capacity of endogenous antioxidants can result in damage to various lipid, protein, and genetic compounds.

Production of ROS is often measured indirectly, using the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Most studies show that this ratio falls during aerobic exercise (e.g. Gohill et al., 1988; Viguie et al., 1993) indicating ROS generation.

A side effect of ROS within the muscle may be acceleration of fatigue. Barclay and Hansel (1991) showed that free radicals enhanced the rate of fatigue of an isolated rodent muscle. Several other studies performed in rodents showed that drugs used to alter cellular GSH influenced rate of fatigue from exercise (Kramer et al 1993, Sen et al 1994a). A single study has an intravenous drug known to increase GSH content in humans; electrically but not voluntary muscle fatigue was delayed (Reid et al, 1994). However, the drug caused multiple side effects and is not well tolerated.

Whey protein isolate may increase glutathione and delay fatigue.

These findings suggest that increasing cellular glutathione could improve the body's ability to squelch ROS and delay fatigue. As an increase in glutathione cannot be achieved through consumption or even intravenous injection, because the compound cannot pass through cell membranes, most efforts to enhance glutathione quantity has focused on boosting the blood concentration of cysteine, as intracellular synthesis of glutathione is limited by the availability of this amino acid. A dietary cysteine donor, whey protein isolate, has been shown in clinical situations to boost plasma glutathione levels between 25 and 44% over just 2 weeks of supplementation (Micke et al, 2001).

A study by Lands et al (1999) showed a 35.5% increase in lymphocyte GSH as well as a 13% increase in peak power and total work performed during a maximal cycling bout of 30 s for subjects consuming 20 g of a whey protein isolate (Immunocal®) for three months. Although these are exciting results with a dietary supplement, there are several questions that remain due to the design and measurements used in that study. For example, the use of a 30 s maximal exercise test is an unusual one to use for an evaluation of a product thought to be effective due to elevation of antioxidant capacity. Most studies do not find that a brief, intense exercise like this is likely to be limited by oxidative products. Oxidation of GSH is not observed with brief, intense exercise (Sastre et al 1992). Although this study validated an increase in total GSH, they did not include a measure of oxidation of GSH caused by the exercise. Thus, the mechanism for an improvement in performance cannot be clearly ascribed to higher GSH. A more appropriate exercise test (i.e. more prolonged) would be one that has been shown by others to cause oxidative stress, as illustrated by oxidation of GSH. In order to identify glutathione boosting as the mechanism for a performance effect, it would be important to measure GSH/GSSG ratio to determine whether this ratio is changed during the exercise and if the change is muted by the Immunocal® supplement.

Whey protein isolate may affect lean tissue mass.

Lands et al. (1999) also reported that the Immunocal® supplement increased lean tissue mass in the healthy men over 3 months. However, the subjects consuming the Immunocal® also reported increased physical activity during the experimental period compared to the placebo, potentially confounding the improved body composition finding. Limited other research supports the potential value of increased blood cysteine on lean tissue. Low plasma cysteine concentrations predicted losses in lean tissue consequent to a weight training program in healthy individuals (Kinscherf et al, 1996). Observations in HIV patients also show that low cysteine concentrations exist prior to tissue wasting (Droge and Holm, 1997; Hack et al, 1997). More

research is warranted to determine the effect of a cysteine donor like whey protein isolate on lean tissue mass.

Many people self-impose a catabolic state with energy restriction to lose weight. Studies from our laboratory have shown that dieting causes loss of lean tissue as measured by net nitrogen loss in the urine (Walberg et al, 1988). The potential value of whey protein isolate on lean tissue loss during energy restriction has not been studied.

Whey protein isolate may affect immunity

Whey protein has been shown by several research groups to enhance the immune system in mice (Parker and Goodrum, 1990; Wong and Watson, 1995). The mechanism for improved immunity is hypothesized to be an increase in glutathione levels in immunocytes. Patients with HIV have reduced glutathione levels, along with impaired immunity. Three months of whey protein supplementation has been shown to improve immune status (i.e. CD4+/CD8+) in such patients (Bounous et al, 1993). Strenuous exercise, in addition to depleting GSH stores via elevated oxygen usage, has been shown to reduce immune response and increases susceptibility to illness in humans (Neiman and Pederson, 1999). Although, whey protein isolate has been used in clinical situations to boost glutathione in order to improve immunity, whether whey protein can reduce the depression in immunity resulting from hard exercise has not been adequately studied.

Other studies have shown a reduction in tissue GSH content during food restriction in rats, suggesting an increased vulnerability to oxidative stress (Leeuwenburg and Ji, 1996). This oxidative stress may be linked to immunity. Limited but provocative studies have shown an impairment of immune response in overweight individuals (Nieman et al, 1996) and athletes (Kono et al, 1988) who underwent energy restriction.

Statement of the problem

In summary, whey protein isolate has been shown to enhance immunity (Bounous et al, 1993), boost blood cysteine, and increase cellular glutathione levels (Bounous, 2000). Although mounting concentrations of ROS have been suggested to accelerate fatigue, increased glutathione levels via whey protein isolate consumption have yet to be adequately investigated as a mechanism for delaying the onset of fatigue or as a way to reduce the depression in immunity resulting from strenuous exercise. While various studies suggest that the elevation of cysteine may reduce loss of lean tissue in catabolic states (Kinscherf et al, 1996), none have investigated whether whey protein consumption may have special value to individuals who are dieting but want to maintain lean body mass. *This study will examine the value of whey protein isolate on glutathione status, exercise, and immunity during energy balance and restriction.*

II. Procedures

Subject selection

Twenty-eight well-trained, healthy men or women (likely cyclists) will be recruited for participation as subjects (eight for the pilot study, twenty for the experimental study). We anticipate recruiting subjects via posted fliers and e-mail announcements. Those who respond will be told of the general plan for the study. Those still interested will be invited to a group or an individual session to hear the details of participation and potential risks. They will be given a chance to ask any questions. Those still interested will receive a copy of the informed consent. Those who return this signed document will be considered during subject selection. Subjects will undergo a health screening and will be excluded using the criteria of the American College of Sports Medicine (ACSM) for "low risk" for exercise participation and testing. According to

ACSM, individuals at low risk can undergo moderate or vigorous exercise without medical evaluation and approval. "Low risk" is defined as men less than 45 years and women less than 55 years who have no symptoms of cardiopulmonary disease and have no more than one risk factor (defined by ACSM) for heart disease (family history, cigarette smoking, hypertension, high blood cholesterol, diabetes, obesity- BMI>30, or sedentary lifestyle). Additional exclusionary criteria related to the low calorie diet and supplement consumption include: eating disorders, food allergy. Subjects who have injuries or orthopedic limitations that affect their ability to do the exercise will be excluded. Finally, those who have a fear of blood withdrawal with needles will not be included in the study (medical screening forms attached).

Pilot study

A pilot study will be performed to determine the amount of time and dose required to significantly boost plasma GSH content with Immunocal® supplementation in this population. Eight subjects (men or women aged 18-25 who participate in regular exercise) will consume Immunocal® each day for 28 days. Four subjects will consume 20 g/d while four will consume 40 g/d of Immunocal. A baseline and weekly blood samples will be taken for analysis of total GSH in whole blood and in lymphocytes. The subjects will be queried for any side effects or difficulties with the protein supplement. The results of this pilot study will determine the dose (20 or 40 g/d) as well as the duration of feeding required to elicit an increase in blood GSH for the experimental trial.

Experimental study

Baseline measurements will be performed on all subjects: aerobic fitness and body composition (see below). Within a week of the baseline aerobic capacity test, each subject will perform a baseline submaximal exercise test designed to stress the oxidative and immunity systems, entailing 45 minutes of cycling on an ergometer at workload determined from the maximal exercise test to elicit 70% of peak oxygen consumption followed by a timed test to exhaustion at 90% of peak oxygen consumption. Oxygen consumption, VO_2, will be measured at the start of the exercise test to verify correct intensity and every 15 minutes thereafter for the duration of the test. Blood samples will be taken prior to the exercise test, after 45 minutes of exercise, within 5 minutes of exhaustion, and 1.5 hours after the completion of exercise.

Following completion of baseline testing, subjects will consume Immunocal or casein (both whey protein and casein are natural proteins found in milk and other dairy products) placebo, in addition to their normal diet, in an amount and for a duration to be determined by the pilot study. One week prior to the completion of the supplementation period, subjects will begin a controlled dietary period. During the controlled dietary period, all subjects will be provided with an energy restricted, formula diet (Ensure High Protein, 20 kcal/kg, 54.7% CHO, 21.3% PRO, 24% Fat) designed to cause weight loss, for 7 days. Each subject will also continue to consume Immunocal or placebo during this period. During this period, subjects will be given containers to collect all of their urine (used to assess body protein loss). They will bring urine collections in daily and receive a new collection container for the next day.

At the end of the 7 day controlled dietary period, subjects will again complete the submaximal exercise test with blood sampling to determine effects of energy restriction and supplementation on performance, antioxidant status, and immunity.

Peak Oxygen Consumption (VO_2 peak) will be determined for all subjects using a graded exercise test on a cycle ergometer (Monark 818E Ergomed C). Prior to VO_2 peak testing subjects will be familiarized with bicycle exercise at a constant pedaling rate while breathing through the mouthpiece and breathing valve. The test will begin at a low intensity, 75 watts, while pedaling at 70 rpm and will increase 25 w every 2 min until the subject can no longer maintain 70 rpm (defined as maximal capacity). Continuous indirect calorimetry will be

performed using a metabolic cart system (Sensormedics). This requires subjects to breath into a mouthpiece during the entire exercise test**.** Their expired air will be analyzed in order to calculate their oxygen consumption. Excluding warm up and cool down, the test will likely last between 10 and 15 minutes.

Body composition will be determined by skinfold measurement at three sites: thigh, chest, and abdomen (men) or tricep, thigh and suprailiac (women). This involves using a caliper to measure the thickness of a fold of skin picked up at the listed sites.

Blood collections will be performed at baseline and weekly during the pilot study (5 samples/subject) and on four occasions (pre-exercise, after 45 minutes of exercise, immediately post-exercise, and 1.5 hours post-exercise) during each of the two submaximal exercise tests during the experimental study (8 samples/subject). The amount in each sample is about four teaspoons (20 ml). Blood samples will be tested for HIV if an experimenter becomes exposed to blood during collection or later during analysis. Blood samples will be analyzed for indicators of oxidative stress (lipid hydroperoxides, glutathione and oxidized glutathione) and immunity [*in vitro* lymphocyte proliferation, lymphocyte populations (CD4, CD8, CD3), phagocytosis and oxidative burst of granuloctyes and monocytes to bacteria challenge].

Urine Collections. Each day of the low calorie diet, subjects will be given several jugs to be used to collect all their urine for the day. They will be asked to bring in the jug(s) to the experimenters each day and pick up more for the next day. The urine volume will be measured and samples analyzed for nitrogen and creatinine to determine body protein change during the weight loss.

III. Risks and Benefits

VO_2 peak testing increases the short-term risk of cardiovascular events such as sudden death and myocardial infarction. The American College of Sports Medicine states that the risk of death during or immediately after a maximal exercise test is less than 0.01% and that the risk of myocardial infarction (heart attack) is less than 0.04% (American College of Sports Medicine Guidelines 2000). As most of the studies that contribute to these statistics have involved testing of individuals at risk of disease, it is likely that testing of the young, healthy subjects in our study is of even lower risk. The subjects we intend to use are at very low risk because of their young age, trained condition, and screening to eliminate those with elevated risk. Subjects selected will be in the "low risk" stratification according to ACSM guidelines (2000), i.e. men less than 40 years of age, asymptomatic of disease, and positive for no more than one of the following risk factors: family history, cigarette smoking, hypertension, hypercholesterolemia, impaired fasting glucose, obesity, and sedentary lifestyle. Subjects will be monitored throughout the exercise tests by the experimenters for signs and symptoms of cardiovascular problems (e.g. abnormal gait, pale, shortness of breath, angina). Fatigue, muscle soreness, and muscle strains could result from the exercise tests but the latter is unlikely due to the high activity level of the subjects and the safety of stationary biking.

Whey protein is consumed marketed for athletes who wish to increase their lean body mass as well as to patient populations (e.g. HIV, cancer). As whey protein is a constituent of foods normally consumed (dairy products) side effects are unusual. Those subjects with allergy to milk will be excluded. Studies in which subjects consumed 20 g/d whey protein for 3 months (Lands et al, 1999) and 45 g/d for 6 months (Micke et al, 2001) reported no severe adverse side effects of consumption. The most common mild side effect was gastrointestinal disturbance. We

will instruct subjects to consume the supplement directly with a meal and water to decrease gastrointestinal problems.

Consumption of a 20 kcal/kg/d formula diet, as will be used in this study, has been used by our group multiple times in the past (e.g. Walberg Rankin et al, 1988, Walberg Rankin et al, 1996, Rockwell et al, 2001) for from 3 to 10 days. We expect that subjects will lose 2-4 kg over the week, although some of this is fluid weight. This is not a dangerous rate of weight loss for healthy subjects over a 7 day period. They may experience fatigue due to weight loss or constipation due to low fiber intake over the 7 d. Appropriate laxatives will be recommended for those experiencing the latter.

Risks of blood withdrawal include bruising and very limited risk of infection. All blood draws will be taken using sterile equipment by a Certified Medical Laboratory Technician (Janet Rinehart) or one of the experimenters trained by Ms. Rinehart (M. Shute) experienced in the procedure. Universal precautions will be taken in collection and handling of all blood samples. Subjects will be told that their blood will be analyzed for presence of HIV if an experimenter is exposed to their blood.

IV. Compensation

Subjects will be financially compensated $50 for completion of the pilot study ($5/wk + $30 for completion) or $100 for completion of the experimental study ($20 for baseline testing + $80 for completion of the study). Additionally, they will receive information on their body composition and aerobic capacity.

V. Confidentiality

The data from this study will be kept strictly confidential. No data will be released to anyone but the principal investigator and graduate students involved in the project without written consent of the subject. Data will be identified by subject number.

Biographical Sketches

Janet Walberg Rankin is a Professor in the Department of Human Nutrition, Foods, and Exercise. She has been on the faculty at Virginia Tech since 1982. She earned her B.S. in Zoology from Duke University in 1977 and her Ph.D. in Nutrition from the University of California at Davis in 1982. Her research is primarily in sports nutrition and weight control for athletes and obese individuals. Publications of her research has appeared in journals such as *International Journal of Sport Nutrition, Medicine and Science in Sports and Exercise, International Journal of Sports Medicine, and Journal of Nutrition Education.*

Korinn Saker is an Assistant Professor in the Department of Large Animal Clinical Sciences at the Virginia/Maryland College of Veterinary Medicine. Her research specialty is the influence of nutrition on immunity.

Michael Houston is Professor and Department Head of Human Nutrition, Foods, and Exercise. He is an exercise biochemist with a research focus is role of antioxidants in exercise. He has written a textbook, Biochemistry Primer for Exercise Sciences, as well as numerous research articles in journals such as *Journal Applied Physiology, Canadian Journal of Applied Physiology, and Journal of Nutrition.*

Max Shute is a graduate student/teaching assistant in the Department of Human Nutrition, Foods, and Exercise. He earned his B.S. in Nutrition from Western Illinois University in 1993 and a Masters in Exercise Science from Appalachian State University in 2000. His research involvement during his Master's program included studies of the immune response to exercise, weight control for the obese, equipment validation. He has just completed a study using most of the equipment planned for use in this study-- effect of conjugated linoleic acid on metabolic rate and fuel use at rest and during exercise.

Sean Heffron is a graduate student/teaching assistant in the Department of Human Nutrition, Foods, and Exercise. He earned his A.B. in Biology from Duke Univeristy in 1998. He has been employed by the Nutrition component of the Duke University Diet and Fitness Center in Durham, North Carolina and by the Yokohama City Board of Education in Yokohama, Japan. He has been an assistant for several studies performed in Dr. Rankin's laboratory over the last year.

References

Barclay JK and Hansel M. Free radicals may contribute to oxidative skeletal muscle fatigue. *Can. J. Physiol. Pharmacol*. 69(2):279-284, 1991.

Bounous G. Whey protein concentrate and glutathione modulation in cancer treatment. *Anticancer Res*. 20(6C):4785-4792, 2000.

Bounous G, Baruchel S, Falutz J, Gold P. Whey proteins as a food supplement in HIV-seropositive individuals. *Clin. Invest. Med.* 16(3):204-209, 1993.

Droge W and Holm E. Role of cysteine and glutathione in HIV infection and other diseases associated with muscle wasting and immunological dysfunction. *FASEB J.* 11:1077-1089, 1997.

Gohil K, Viguie C, Stanley W, Brooks G, Packer L. Blood glutathione oxidation during human exercise. *J. Appl. Physiol*. 64(1):115-119, 1988.

Hack V, Schmid D, Breitkreutz R, Stahl-Henning C, Drings P, Kinscherf R, Taut F, Holm E, Droge W. Cystine levels, cystine flux, and protein catabolism in cancer cachexia, HIV/SIV infection, and senescence. *FASEB J.* 11:84-92, 1997.

Kramer K, Dijikstra H, Bast A. Control of physical exercise of rats in a swimming basin. *Physiol. Behav*. 53(2):271-276, 1993.

Kinscherf R, Hack V, Fischbach T, Freidmann B, Weiss C, Edler L, Bartsch P, Droge W. Low plasma glutamine in combination with high glutamate levels indicate risk for loss of body cell mass in healthy individuals: the effect of N-acetyl-cysteine. *J. Mol. Med*. 74(7):393-400, 1996.

Kono, I, H. Kitao, M Matsuda, S. Haga, H. Fukushima, H. Kashiwagi. Weight reduction in athletes may adversely affect the phagocytic function of monocytes. *Phys. Sportsmed*. 16:56-65, 1988.

Lands LC, Grey VL, Smountas AA. Effect of supplementation with a cysteine donor on muscular performance. *J. Appl. Physiol*. 87(4):1381-1385, 1999.

Leeuwenburgh C and Ji LL. Alteration of glutathione and antioxidant status with exercise in unfed and refed rats. *J. Nutr*. 126:1833-1843, 1996.

McArdle A, Pattwell D, Vasilaki A, Griffiths RD, Jackson MJ. Contractile activity-induced oxidative stress: cellular origin and adaptive responses. *Am. J. Physiol. Cell Physiol*. 280(3):C621-627, 2001.

Micke P, Beeh KM, Schlaak JF, Buhl R. Oral supplementation with whey proteins increases plasma glutathione levels of HIV-infected patients. *Eur. J. Clin. Invest.* 31(2):171-178, 2001.

Nieman DC and Pedersen BK. Exercise and immune function. Recent developments. *Sports Med*. 27(2):73-80, 1999.

Nieman DC, Nehlsen-Cannarella SL, Henson DA, Butterworth DE, Fagoaga OR, Warren BJ, Rainwater MK. Immune response to obesity and moderate weight loss. *Int. J. Obesity* 20:353-360, 1996.

Parker, N.T. and K.J. Goodrum. A comparison of casein, lactalbumin, and soy protein effect on the immune system. *Proc. Nutr. Soc*. 57:43-47, 1998.

Rankin JW, Ocel JV, Craft LL. Effect of weight loss and refeeding diet composition on anaerobic performance in wrestlers. *Med. Sci. Sports Exerc*. 28(10):1292-1299, 1996.

Reid MB, Stokic DS, Koch SM, Khawli FA, Leis AA. N-Acetylcysteine inhibits muscle fatigue in humans. *J. Clin. Invest.* 94:2468-2474, 1994.

Rockwell JA, Rankin JW, Toderico B. Creatine supplementation affects muscle creatine during energy restriction. *Med Sci. Sports Exerc.* 33(1):61-68, 2001.

Sastre J, Asensi M, Gasco E, Pallardo FV, Ferrero JA, Furukawa T, Vina J. Exhaustive physical exercise causes oxidation of glutathione status in blood: prevention by antioxidant administration. *Am. J. Physiol*. 263:R992-R995, 1992.

Sen CK, Atalay M, Hanninen O. Exercise-induced oxidative stress: glutathione supplementation and deficiency. *J. Appl. Physiol*. 77(5):2177-2187, 1994.

Sen CK, Rankinen T, Vaisanen S, Rauramaa R. Oxidative stress after human exercise: effect of N-acetylcysteine supplementation. *J. Appl. Physiol*. 76(6):2570-2577, 1994.

Viguie CA, Frei B, Shigenaga MK, Ames BN, Packer L, Brooks GA. Antioxidant status and indexes of oxidative stress during consecutive days of exercise. *J. Appl Physiol*. 75(2):566-572, 1993.

Walberg JL, Leidy MK, Sturgill DJ, Hinkle DE, Ritchey SJ, Sebolt DR. Macronutrient content of a hypoenergy diet affects nitrogen retention and muscle function in weight lifters. *Int. J. Sports. Med*. 9(4):261-266, 1988.

Walberg Rankin, J., J. V. Ocel, L. L. Craft. Effect of weight loss and refeeding diet composition on anaerobic performance in wrestlers. *Med.Sci. Sports Exerc.* 28:1292-1299, 1996.

Walberg-Rankin, J., C. E. Hawkins, D. S. Fild, D. R. Sebolt. The effect of oral arginine during energy restriction in male weight trainers. *J. Str.Cond. Res.* 8 (13): 170-177, 1994.

Wong CW and Watson DL. Immunomodulatory effects of dietary whey proteins in mice. *J. Dairy Res*. 62(2):359-368, 1995

Appendix F. Informed Consent for Participants of Investigative Projects

Informed Consent for Participants of Investigative Projects
Department of Human Nutrition, Foods, and Exercise
Virginia Tech

TITLE: Value of Whey Protein Concentrate on Glutathione Status, Exercise Performance, and Immunity in Men During Energy Balance and Restriction.

PRINCIPAL INVESTIGATORS: Janet Walberg Rankin, Ph.D., Korinn Saker, Ph.D. D.V.M., Michael Houston Ph.D., Max Shute, MS., Sean Heffron, AB.

PURPOSE: Cysteine is a naturally occurring amino acid found in large amounts in the whey portion of milk. Cysteine is the limiting factor in the body's synthesis of glutathione, a natural antioxidant n the body. Several studies have shown that consumption of whey protein increases blood levels of glutathione, a natural compound our body uses to protect itself from damage due to oxygen. Oxygen can damage cells in our bodies (oxidative stress) and the resulting damage has been implicated in a variety of conditions such as cancer, immunity, and heart disease. As oxygen damage can be elevated with increased use of oxygen, exercise increases the concentration of these molecules in the body. High oxidative stress has been hypothesized to contribute to muscle fatigue. Thus, it is possible that increasing concentration of natural body antioxidants such as glutathione will improve exercise performance.

Low levels of cysteine in the blood have been linked to loss of muscle mass in individuals that have poor immunity (e.g. HIV) or in those undergoing strenuous physical activity. Also, Lands et al (1999) reported increased muscle mass with three months of whey protein supplementation. However, it is hard to be sure that this was due to the whey protein because the subjects also increased their physical activity during the experimental period. So, more research must be done to determine if consumption of whey protein isolate improves body composition.

Finally, strenuous exercise has been shown to reduce immunity and increase susceptibility to illness in humans. Whey protein isolate has been used in clinical situations to boost glutathione in patients in order to improve immunity. It has yet to be determined, however, whether whey protein can reduce the depression in immunity resulting from hard exercise. Thus, this study will examine the value of whey protein concentrate on glutathione status, exercise performance, and immunity while maintaining your weight and after one week of weight loss.

Experimental Study

General Design

We will first ask you to complete a medical screening form to make sure that you would not be at elevated risk as a participant in the study. If you are chosen and agree to participate in the study, we will do some baseline measurements that include your body fat (measure the thickness of fat folds at three areas of your body: chest, abdomen, and thigh) and aerobic fitness (see below). Within one week of the completion of these measurements, you will return to the lab for a baseline submaximal exercise test (see below). You will then consume 40 grams of either Immunocal® (whey protein isolate) or

placebo (casein, another protein found in milk) daily (you will not know which treatment you receive) for three weeks. One week prior to the completion of supplementation period, you will begin a formula diet that we will provide. This diet will consist of 20kcal/kg Ensure High Protein® (a meal replacement found at grocery stores) daily. During the week you consume this diet, you will collect all of your urine in containers provided by us. At both the beginning and end of this 7 day controlled diet period you will return to the lab and again complete the submaximal exercise test. At each of the submaximal exercise trials we will take 4 blood samples over approximately 3 hours, and make multiple measures of your exercise intensity with a machine that measures your expired air. In addition, on the first and last days of the controlled diet period you will report to the lab early in the morning in a fasted state for one blood sample. Following this sample, you will consume 20 grams of supplement and remain in the lab for 90 minutes until a second blood sample is taken.

Maximum oxygen consumption testing: This test will measure your aerobic fitness. You will cycle at a desired rpm on a stationary bike starting at a comfortable resistance (75 watts) for 2 minutes. The resistance will increase 25 watts every 2 minutes until exhaustion or you can no longer maintain 60rpm. During this test you will be breathing room air through a mouth-piece similar in nature to a snorkel.

Blood collection: A blood sample will be taken prior to the submaximal exercise tests, after 45 minutes of exercise, within 5 minutes of exhaustion, and 1.5 hours after the completion of exercise. Blood samples will also be taken prior to and following the controlled diet period, during the morning hours, in a fasted state and 90 minutes following consumption of 20 g supplement. The amount in each sample is about two teaspoons. All blood sampling will be performed by a Certified Medical Laboratory Technician, Janet Rinehart, or an experimenter trained by Ms. Rinehart (M. Shute) experienced in the procedure. Your blood will be measured for indicators of oxidative stress (i.e. amount of one of the body's antioxidants, glutathione, that has been modified by oxygen; lipids damaged by oxygen), immunity (e.g. ability of white blood cells to respond to an infectious protein, numbers of specific types of white blood cells), and levels of amino acids. Your blood sample will be tested for HIV if an experimenter becomes exposed to your blood during collection or later during analysis.

Submaximal exercise test: You will pedal on a stationary cycle for 45 minutes at a moderate-high intensity (about 70% of your maximum fitness level). You will then cycle at a high intensity (90% of your maximum fitness level) until you are exhausted and no longer able to maintain the set intensity. As described above, blood and expired air samples will be conducted several times during the exercise test.

Diet: During the formula diet period, we ask that you do not consume any other foods or beverages that contain calories. You may drink as much water or noncaloric beverages (i.e. diet soda, black coffee, unsweetened tea) as you like.

SUMMARY OF SUBJECT RESPONSIBILITIES

1. Refrain from taking any other nutritional supplements without checking first with the experimenters.
2. Give maximal effort on performance tests.
3. Inform the experimenters if you experience any unusual symptoms from any of the testing, or supplements.
4. Inform the researchers of any known medical conditions or allergies you are aware of prior to the study as well as any transmittable diseases acquired during the study.
5. Refrain from eating and/or drinking any caloric foods and/or beverages during the 7-day formula diet period.
6. Collect all urine produced for each of the 7 days of the formula diet period.

RISKS OF PARTICIPATION:

1. Studies in which subjects consumed 20 g/d whey protein for 3 months and 45 g/d for 6 months reported no severe adverse side effects of consumption. The most common mild side effect was gastrointestinal disturbance.
2. Consumption of a 20 kcal/kg/d formula diet, as will be used in this study, has been used by our group multiple times in the past for from 3 to 10 days. We expect that you will lose 2-4 kg over the week, although some of this is fluid weight. You may be tired as a result of the weight loss. As there is little fiber in the formula diet, you may experience some constipation during the weight loss week. We can recommend an appropriate laxative if you experience this.
3. Fatigue, muscle soreness, muscle strains or pulls may result from the aerobic fitness and sub-maximal exercise testing.
4. You may experience some bruising at the site of the blood withdrawal. There is a remote chance of infection as a result of the needle stick, but this is very unlikely due to the use of sterile supplies.
5. There is a remote risk of cardiovascular complications from maximal exercise testing. The American College of Sports Medicine states that the risk of death during or immediately after a maximal exercise test is less than 0.01% and that the risk of myocardial infarction (heart attack) is less than 0.04% (American College of Sports Medicine Guidelines 2000). As most of these studies that contribute to these statistics have involved testing of individuals at risk of disease, it is likely that testing of the young, healthy subjects in our study is of even lower risk.
6. The University will not be responsible for any medical expenses you may have unless the University has been negligent.

BENEFITS OF PARTICIPATION

Your participation will provide you with:

1. Data on your body composition and aerobic fitness.

COMPENSATION

We will pay you $20 for completion of all baseline testing and $80 additional for completion of the experimental study.

ANOYNMITY AND CONFIDENTIALITY
The data from this study will be kept strictly confidential. No data will be released to anyone but those working on the project without your written permission. Data will be identified by subject numbers, without anything to identify subjects by name.

FREEDOM TO WITHDRAW
You are free to withdraw at any time from the study for any reason. Circumstances may come up that the researcher will determine that you should not continue as a subject in the study. For example, lack of compliance to diet or exercise, failure to attend testing sessions and illness could be reasons to have the researchers stop your participation in the study.

APPROVAL OF RESEARCH
This research has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech, and by the Department of Human Nutrition, Foods, and Exercise. You will receive a copy of this form to take with you.

SUBJECT PERMISSION
I have read the informed consent and fully understand the procedures and conditions of the project. I have had all my questions answered, and I hereby give my voluntary consent to be a participant in this research study. I agree to abide by the rules of the project. I understand that I may withdraw from the study at any time.

If I have questions, I will contact:
- Principal Investigator: Janet Walberg Rankin, Professor, Department of Human Nutrition, Foods, and Exercise. 231-6355
- Chairman, Institutional Review Board for Research Involving Human Subjects: David Moore, 231-4991
- Department reviewer: Robert Grange, 231-2725

Name of Subject (please print)_____

Signature of Subject_____ Date_____

Appendix G: Instructions for Subjects

FILLING OUT YOUR FOOD RECORD

Please follow the directions for filling out your diet record carefully. The more accurate your record, the more complete your dietary analysis and recommendations will be.

You will be completing a 3-day food record. Two days should be weekdays, one a weekend. Please record your name, day of the week and the date on which you completed your record on the top of each food record form.

Time: List the time you consumed the food or beverage

Food/Beverage: Write the name of the food or beverage

Amount: List the amount eaten or drunk in ounces (oz), teaspoons (tsp), tablespoons (tbsp), cups (c), in fractions of the aforementioned units, or in units such as "1 slice of whole wheat bread," "1 raw orange," etc (See Sample Food Record)

Description/Preparation: Describe methods used to prepare the food or beverage. Examples: baked, steamed, canned, fried, fresh, raw, frozen, with sauce, etc. Include brand names or restaurant names.

REMEMBER! Did you record all snacks you ate each day?
Did you record all beverages you drank? Such as: coffee, tea, water, milk, soda, juice, alcohol, etc.
Did you record any cream, milk or sugar added to your coffee or tea?
Did you record any lettuce, tomato, onion, etc. added to any sandwiches?
Did you record all condiments used? Such as: mayo, mustard, ketchup, salsa, sour cream, salad dressing.
Did you add butter or margarine to your food? Is it recorded?
Did you describe how each food was prepared (e.g. baked, fried, broiled, steamed or boiled)?

Amount Estimation Tools: One teaspoon = size of thumb above the joint
One cup = size of fist or walkman
One ounce cheese = size of thumb
3 oz meat = size of deck of cards
½ cup = size of tennis ball

NAME: _____ DAY: _____ DATE: _____

Food Record

Time	Food/Beverage	Amount	Preparation/Description
8 AM	Cereal	1 cup	Cheerios
	milk	½ cup	2 %
	sugar	2 teaspoons	
	banana	1	large
	orange juice	8 oz.	From concentrate
12:00 noon	sandwich	1	
	bread	2 slices	Wonder, white
	turkey	3 slices	Oscar Mayer
	cheese	1 slice	Kraft, processed swiss
	lettuce	2 leaves	Romaine lettuce
	mayonnaise	1 tablespoon	Kraft mayo, low fat
	apple	1	medium
	cookie	2	medium, chocolate-chip
	ice tea	16 oz	sweet tea
2:45 pm	candy bar	1	Snickers
6 pm	spaghetti	~2.5 cups	
	spaghetti sauce	.5 cup	Tomato & Meat sauce
	grated cheese	2 tablespoons	Parmesan

NAME: _____ DAY: _____ DATE: _____

Food Record

Time	Food/Beverage	Amount	Preparation/Description
9 am	cereal	bowl use cups	with milk what kind of cereal?
	Add milk here	how much milk?	Whole, skim, 1%???
	apple	1	raw small, med, large???
10:30 am	juice	1 bottle how many oz?	Apple Brand name? Fresh?
12:30 pm	hamburger	1 how much meat?	Brand name?
	List condiments!		
	coke	Big Gulp how many oz?	
	broccoli	½ cup	how prepared? Toppings?
3:30 pm	candy bar	1	snickers
6:30 pm	spaghetti	2 cups	with sauce list sauce separately
	what kind of sauce?	how much sauce?	
	salad list salad ingredients separately	1 bowl	with dressing list dressing separately
	what kind of dressing?	How much dressing?	
	cookies	3	What kind of cookies? What size????

NAME: _____ DAY: _____ DATE: _____

Food Record

Time	Food/Beverage	Amount	Preparation/Description

Formula Diet Instructions

The purpose of the formula diet is to control energy, as well as nutrient, intake. The diet will supply 20 kcal per kg body weight (1 kg is 2.2 pounds), so a 165 pound person will consume 1500 kcal per day. This is not a starvation diet. Food will be supplied to you each day in the form of Ensure, a nutritional supplement that is 55% carbohydrate, 21% protein and 24% fat. The diet will provide all of the recommended amounts of vitamins and minerals, so vitamin supplementation is not necessary.

You can expect to lose about 1-3 pounds of body fat, and even more water weight during the energy restriction period. You are likely to regain the weight, depending on what you eat, once the experiment is over. Drink plenty of water, at least six cups per day or more. Since weight loss can result in dehydration, you may feel tired and dizzy. Consuming enough water will prevent this from occurring.

It is very important that you consume all of the ENSURE that you are given each day, and that you do not consume any other calories.
Some subjects will be given a plastic container holding a small amount of ENSURE along with cans of the beverage. You must bring this container with you every time you come to pick up more ENSURE. Unopened cans do not need to be refrigerated but once opened should be stored in the refrigerator until consumption.

It is OK to consume non-caloric products while on the diet, such as:

- Water
- Diet soft drinks
- Coffee (artificial sweeteners such as Equal and Sweet-n-Low only, no milk or artificial creamers)
- Tea (artificial sweeteners only)
- Sugarless gum (up to 3 pieces per day)

If you think there is something else that could be OK to eat during the diet, check with the investigators first. The ENSURE tastes best cold, so it is suggested you store it in the refrigerator.

Expect to feel some fatigue on the diet, and you may also experience a change in your bowel habits. This is normal. ***Let us know if you experience any unusual symptoms.***

Instructions for Urine Collection

Urine analysis will be performed on all urine voided during the four-day formula diet, weight loss period. You will collect urine produced over each of the four 24-hour periods separately and bring to us in the laboratory in 230 War Memorial Hall. Our analysis of the urine will tell us how much lean tissue you lose during the weight loss (we will provide this information to you after analysis if you choose). So, if you do not provide us with full collection, our values will not be correct. Please make sure you make every effort to collect all urine over this period but let us know if you accidentally missed any collections.

The first collection period ***does not include*** the first voiding on the day before testing, but will begin ***immediately after*** the first voiding. The final collection period will end after the first voiding the morning after the fourth day of the formula diet, weight loss period.

You will be given three 1-liter containers each day in which to collect your urine. There will be a small amount of hydrochloric acid in each bottle as a preservative. This is acid, so avoid skin contact. All urine should be voided directly into the collection bottle. Fill one collection bottle before using another bottle. Once a bottle is full, please refrigerate until the bottle can be returned. We suggest you carry a bottle with you when you leave the house in a plastic opaque bag.

Collection bottles are to be turned in at around 8 AM (7:30AM-9:00AM) to 230 War Memorial Hall during each day of collection.

Instructions for Protein Consumption

You will be consuming 40 grams of either whey or casein protein per day, each day of the study. Neither you, nor the experimenters, will know which of the proteins you will receive (Dr. Rankin has this information and will break the key at the end of the study). This protein will be provided to you in plastic baggies holding 20 grams each. We ask that you consume ***one package in the morning*** and ***one in the evening*** each day of the study.

You will be given a one-week supply (14 packages) following your first exercise test. Another one-week supply will be picked up in the morning, exactly one week after completing the first exercise test, from the lab in 230 War Memorial Hall. After you have taken the supplement for two weeks, you will begin the energy restriction portion of the study. During this time, you will receive your protein supplement at the same time you come to the lab to pick-up your formula, weight loss diet, ENSURE, and drop off your urine containers.

You will be given a compliance form to mark each time you consume the supplement. Please be honest in marking this form. While it is very important that you take all of the protein, we need to know if you were unable to consume the supplement for some reason. Please also record on this form any symptoms you may experience which you feel can be attributed to the protein supplement.

The protein you will be consuming does not easily dissolve. You will be given a list of suggested ingestion methods which you may like to try. We have improved solubility by allowing the protein to sit in orange juice for 10-15 minutes before stirring and drinking. Please let us know if you come up with any "favorite recipes." ***IT IS VERY IMPORTANT THAT YOU DO NOT USE A BLENDER OR HEAT THE PROTEIN SUPPLEMENT!*** This reduces the effectiveness of the supplement. Brisk stirring with a spoon or fork is allowed. You should consume the supplement within 30 minutes of mixing.

Enjoy!

Appendix H: Baseline VO_{2peak} Testing Data Sheet

Immunocal Study Peak Oxygen Consumption Testing
Data collection sheet

Subject name: _____ ID# _____ Date: - - ___

Age: _____ Ht: _____cm Wt: _____kg Ambient Temp: _____

Minute	watts	RPE	VO_2
1	100		
1:30	115		
2	130		
2:30	145		
3	160		
3:30	175		
4	190		
4:30	205		
5	220		
5:30	235		
6	250		
6:30	265		
7	280		
7:30	295		
8	310		
8:30	325		
9	340		
9:30	355		
10	370		
10:30	385		
11	400		
11:30	415		
12	430		
12:30	445		
13:00	460		
13:30	475		
14	490		

RelativeVO_{2pk}: _____ml/kg/min
70% VO_{2pk}: _____, watts = _____
90% VO_{2pk}: _____, watts = _____

Appendix I: Endurance Exercise Test Data Sheet

Test day data collection sheet

Subject #_____ Wt:_____kg Date:___/___/___

Test #_____

Time of start of exercise:_____

70% = _____watts 70% = _____ml/kg/min

90% = _____watts 90% = _____ml/kg/min

VO_2 check 1:_____

VO_2 check 2:_____

VO_2 check 3:_____

90% VO_2 time to exhaustion:_____

Wingate Test

Resistance: .09kg per kg body wt. = _____

Watts = load (kg) x revolutions x 11.765

5-second rpm counts: _____ _____ _____ _____ _____ _____

Peak power (greatest power in 5-sec period, usually the 1st 5-sec) = _____w

Avg. power for the six 5-second periods = _____w

Fatigue rate: 100 x (peak power – lowest power)/peak power = _____%

Appendix J: Protein Consumption Compliance Sheet

PROTEIN STUDY
SUBJECT COMPLIANCE FORM

SUBJECT NUMBER: _____ WEEK NUMBER: _____

Please record the number of packets of whey protein you consume during the week by making a mark in one square per packet per day. While it is important that you consume all of the protein you are given for the week, if you forget to consume a packet, please do not make a mark on the form. An accurate record of your total consumption is of utmost importance.

If you forget to consume a packet one day, please consume that packet, in addition to your normal daily dose, on the next day and mark the boxes accordingly.

Use the comment area to report any problems you have with the supplement or side effects (positive or negative) you feel it is producing.

DAY	**PROTEIN PACKETS**					**COMMENTS**
Friday						
Saturday						
Sunday						
Monday						
Tuesday						
Wednesday						
Thursday						

Additional Comments:

Appendix K: Exit Survey

Immunocal Study Exit Survey

Name: _____ Subject #_____

Congratulations! You have completed the study. Before you leave we would like you to answer a few questions. You cannot be penalized for your responses so please be COMPLETELY honest. We enjoyed your participation and hope you will consider other study opportunities that may come along from the HNFE Dept.

1. During the 4 days of the Ensure diet, did you eat any other foods which contained calories? If so, please indicate when you ate, what items, and how much.

2. Did you consume 100% of your supplement throughout the study? If not, please indicate how much supplement was not taken and when.

3. Which supplement do you think you had, Immunocal or Placebo?

4. On a scale of 1-10 (10 being extremely difficult), how difficult was the energy restriction period?

5. Do you think the supplement had any positive or negative effects on you mentally and/or physically? If so, briefly describe.

6. Please provide any comments on how the study was run and any ways we could have been more helpful or done a better job.

7. Do you want your body composition, fitness, and performance information from the study? (please provide your summer address for us to send this information at the end of the study)

Your $100 check should arrive after the next VT pay period. Please contact Max Shute (mshute@vt.edu) or Dr. Rankin (jrankin@vt.edu) if you do not receive the check within 4-5 weeks. Thanks and good luck in the future!

Appendix L: Recruitment Fliers

Department of Human Nutrition, Foods and Exercise
338 Wallace Hall
Virginia Tech
Blacksburg, Virginia 24061

It's not the draft!

MALES AGED 18-25

IT'S A PROTEIN SUPPLEMENTATION STUDY!!

Participation will involve several weeks of protein supplementation and once weekly blood tests. Participants will receive financial compensation.

FOR MORE INFO, and to see if you qualify, please contact:

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Max Shute maxshute@hotmail.com

Make **money** while you study!!!

(and we study you)

If you are:

- **Male**
- **Aged 18 – 25**
- **Available Spring Semester**

You may qualify to participate!

Testing will involve resting (during which you can study) and exercise tests. We'll also take a little of your blood (don't worry, you have plenty!).

FOR MORE INFO, please contact:

Max Shute

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Max Shute maxshute@hotmail.com
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Calling all cyclists!

to participate in a

Nutritional Supplement Study

Department of Human Nutrition, Foods and Exercise
338 Wallace Hall
Virginia Tech
Blacksburg, VA 24061

If you are:
- **Male**
- **Aged 18 – 25**

You may qualify to participate!

Participation will involve several weeks of whey protein supplementation, a weeklong diet period, exercise tests and blood tests and will be rewarded with financial compensation.

FOR MORE INFO, please contact:

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REFERENCES

1. Abumrad, N. N. and B. Miller. The physiologic and nutritional significance of plasma-free amino acid levels. *JPEN.* 7:163-170, 1983.
2. Agin, D., D. Gallagher, J. Wang, S. B. Heymsfield, R. N. Pierson Jr., and D. P. Kotler. Effects of whey protein and resistance exercise on body cell mass, muscle strength, and quality of life in women with HIV. *AIDS.* 15:2432-2440, 2001.
3. Anthony, J. C., T. G. Anthony, S. R. Kimball, and L. S. Jefferson. Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. *J. Nutr.* 131:856S-860S, 2001.
4. Antonio, J., M. S. Sanders, and D. Van Gammeren. The effects of bovine colostrums supplementation on body composition and exercise performance in active men and women. *Nutrition.* 17: 243-247, 2001.
5. Amrstrong, M. D. and U. Stave. A study of plasma free amino acid levels II. Normal values for children and adults. *Metabolism.* 22:561-569, 1973.
6. Barclay, J. K. and M. Hansel. Free radicals may contribute to oxidative skeletal muscle fatigue. *Can. J. Physiol. Pharmacol*. 69:279-284, 1991.
7. Bergstrom, J., P. Furst, and E. Vinnars. Effect of a test meal, without and with protein, on muscle and plasma free amino acids. *Clin. Sci.* 79:331-337, 1990.
8. Bidlingmeyer, B. A., S. A. Cohen, and T. L. Tarvin. Rapid analysis of amino acids using pre-column derivatization. *J. Chrom.* 336:93-104, 1984.
9. Bingham, S. A. and J. H. Cummings. The use of creatinine output as a check on the sompleteness of 24-hour urine collections. *Hum. Nutr.: Clin. Nutr.* 39C:343-353, 1985.
10. Biolo, G., K. D. Tipton, S. Klein, and R. R. Wolfe. An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *Am. J. Physiol.* 273:E122-129, 1997.
11. Blomstrand, E. Amino acids and central fatigue. *Amino Acids.* 20:25-34, 2001.
12. Bogardus, C., B. M. LaGrange, E. S. Horton, and E. A. Sims. Comparison of carbohydrate-containing and carbohydrate-restricted hypocaloric diets in the treatment of obesity. Endurance and metabolic fuel homeostasis during strenuous exercise. *J. Clin. Invest.* 68:399-404, 1981.

13. Boirie, Y., M. Dangin, P. Gachon, M-P. Vasson, J-L. Maubois, and B. Beaufrere. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc. Natl. Acad. Sci. USA*. 94:14930-14935, 1997.

14. Bounous, G. Whey protein concentrate and glutathione modulation in cancer treatment. *Anticancer Res*. 20:4785-4792, 2000.

15. Bounous, G. and P. A. L. Konghsvn. Influence of protein type in nutritionally adequate diets on the development of immunity. In Absorption and Utilization of Amino Acids, Volume II. ed. M. Friedman, CRC Press, Boca Raton FL, pp 219-233, 1989.

16. Bridgeman, M. M., M. Marsden, W. MacNee, D. C. Flenley, and A. P. Ryle. Cysteine and glutathione concentrations in plasma and bronchoalveolar lavage fluid after treatment with N-acetylcysteine. *Thorax*. 46:39-42, 1991.

17. Brinkworth, G. D., J. D. Buckley, P. C. Bourndon, J. P. Gulbin, and A. David. Oral bovine colostrum supplementation enhances buffer capacity but not rowing performance in elite female rowers. *Int. J. Sport Nutr. Exerc. Metab.* 12:349-365, 2002.

18. Brodan, V., E. Kuhn, J. Pechar, and D. Tomkova. Changes of free amino acids in plasma of healthy subjects induced by physical exercise. *Europ. J. Appl. Physiol.* 35:69-77, 1976.

19. Buckley, J. D., M. J. Abbott, G. D. Brinkworth, and P. B. Whyte. Bovine colostrum supplementation during endurance running training improves recovery, but not performance. *J. Sci. Med. Sport.* 5:65-79, 2002.

20. Burke, D. G., P. D. Chilibeck, K. S. Davidson, D. G. Candow, J. Farthing, and T. Smith-Palmer. The effect of whey protein supplementation with and without creatine monohydrate combined with resistance training on lean tissue mass and muscle strength. *Int. J. Sport Nutr. Exerc. Metab.* 11:349-364, 2001.

21. Cahill, G. F., T. T. Aoki, and R. J. Smith. Amino acid cycles in man. *Curr. Top. Cell. Reg.* 18:389-399, 1981.

22. Calbet, J. A. and D. A. MacLean. Plasma glucagon and insulin responses depend on the rate of appearance of amino acids after ingestion of different protein solutions in humans. *J Nutr.* 132:2174-2182, 2002.

23. Clare, D. A. and H. E. Swaisgood. Bioactive milk peptides: A prospectus. *J. Dairy Sci.* 83:1187-1195, 2000.

24. Coombes, J. S., M. Conacher, S. K. Austen, and P. A. Marshall. Dose effects of oral bovine colostrum on physical work capacity in cyclists. *Med. Sci. Sports Exerc.* 34:1184-1188, 2002.

25. Daly, J. N., M. Mihranian, J. Kehoe, and M. Brennan. Effects of postoperative infusion of branched chain amino acids on nitrogen balance and forearm muscle substrate flux. *Surgery.* 94:151-158, 1983.

26. Dangin, M., Y. Boirie, C. Guillet, and B. Beaufrere. Influence of the protein digestion rate on protein turnover in young and elderly subjects. *J. Nutr.* 132:3228S-3233S, 2002.

27. Darling, P. B., M. Dunn, G. Sarwar, S. Brookes, R. O. Ball, and P. B. Pencharz. Threonine kinetics in preterm infants fed their mothers' milk or formula with various ratios of whey to casein. *Am. J. Clin. Nutr.* 69:105-114, 1999.

28. Davies, K. J., A. T. Quintanilha, G. A. Brooks, and L. Packer. Free radicals and tissue damage produced by exercise. *Biochem. Biophys. Res. Commun.* 107:1198-1205, 1982.

29. Decombaz, J., P. Reinhardt, K. Anatharaman, G. von Glutz, J. R. Poortmans. Biochemical changes in a 100 km run: free amino acids, urea, and creatinine. *Eur J Appl Physiol Occup Physiol*. 12:61-72, 1979.

30. Demling, R. H. and L. DeSanti. Effect of a hypocaloric diet, increased protein intake and resistance training on lean mass gains and fat mass loss in overweight police officers. *Ann. Nutr. Metab.* 44:21-29, 2000.

31. Droge, W. and E. Holm. Role of cysteine and glutathione in HIV infection and other diseases associated with muscle wasting and immunological dysfunction. *FASEB J.* 11:1077-1089, 1997.

32. Eriksson, L. S. Splanchnic exchange of glucose, amino acids and free fatty acids in patients with chronic inflammatory bowel disease. *Gut.* 24:1161-1168, 1983.

33. Esmarck, B., J. L. Andersen, S. Olsen, E. A. Richter, M. Mizuno, and M. Kjaer. Timing of postexercise protein intake is important for muscle hypertrophy with resistance training in elderly humans. *J. Physiol.* 535:301-311, 2001.

34. Fisler, J. S., E. J. Drenick, N. N. Yoshimura, and M. E. Swendseid. Plasma concentration of amino acids in obese men consuming very-low-calorie diets composed of soy or collagen protein. *Int. J. Obesity* 9:335-346, 1985.

35. Fogelholm, M. and H. Hiilloskorpi. Weight and diet concerns in Finnish female and male athletes. *Med. Sci. Sports. Exer.* 31:229-235, 1999.

36. Forbes, G. B. and G. J. Bruining. Urinary creatinine excretion and lean body mass. *Am. J. Clin. Nutr.* 29:1359-1366, 1976.

37. Forslund, A. H., L. Hambraeus, H. Van Beurden, U. Holmback, A. E. El-Khoury, G. Hjorth, R. Olsson, M. Stridsberg, L. Wide, T. Akerfeldt, M. Regan, and V. R. Young. Inverse relationship between protein intake and plasma free amino acids in healthy men at physical exercise. *Am. J. Physiol. Endocrinol. Metab.* 278:E857-E867, 2000.

38. Fruend, H., H. Hoover, S. Atamian, and J. Fisher. Infusion of branched chain amino acids in postoperative patients. *Ann. Surg.* 190:18-23, 1979.

39. Fruhbeck, G. Slow and fast dietary proteins. *Nature.* 391:843-4, 1998.

40. Gatti, E., M. Porrini, D. Noe, R. Crovetti, and G. Testolin. Plasma amino acids changes in obese patients on very low-calorie diets. *Int. J. Vitam. Nutr. Res.* 164:81-85, 1994.

41. Gaull, G. E., D. K. Rassin, N. C. Raiha, and K. Heionen. Milk protein quantity and quality in low-birth-weight infants. III. Effects on sulfur amino acids in plasma and urine. *J. Pediatr.* 90:348-355, 1977.

42. Gazzaniga, A. B., K. Waxman, A. T. Day, R. R. Bradford, J. A. Vanderhoof, B. Bivins, and E. Steiger. Nitrogen balance in adult hospitalized patients with the use of a pediatric amino acid model. *Arch. Surg.* 123:1275-1279, 1988.

43. Gibson, R. A. Principles of Nutritional Assessment. New York, NY: Oxford University Press, 1990.

44. Ha, E. and M. B. Zemel. Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people. *J. Nutr. Biochem.* 14:251-258, 2003.

45. Hack, V., D. Schmid, R. Breitkreutz, C. Stahl-Henning, P. Drings, R. Kinscherf, F. Taut, E. Holm, and W. Droge. Cystine levels, cystine flux, and protein catabolism in cancer cachexia, HIV/SIV infection, and senescence. *FASEB J.* 11:84-92, 1997.

46. Hall, W. L., D. J. Millward, S. J. Long, and L. M. Morgan. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *Br. J. Nutr.* 89: 239-248, 2003.

47. Hendler, R. and A. A. Bonde. Very-low-calorie diets with high and low protein content: impact on triiodothyronine, energy expenditure, and nitrogen balance. *Am. J. Clin. Nutr.* 48:1239-1247, 1988.

48. Heymsfield, S. B., C. Arteaga, C. McManus, J. Smith, and S. Moffitt. Measurement of muscle mass in humans: validity of the 24-hour urinary creatinine method. *Am. J. Clin. Nutr.* 37:478-494, 1983.

49. Heymsfield, S. B., C. McManus, J. Smith, V. Stevens, and D. W. Nixon. Anthropometric measurement of muscle mass: revised equations for calculating bone-free arm muscle area. *Am. J. Clin. Nutr.* 36:680-690, 1982.

50. Hildebrandt, W., R. Kinscherf, K. Hauer, E. Holm, W. Droge. Plasma cystine concentration and redox state in aging and physical exercise. *Mech. Age Dev.* 123:1269-1281, 2002.

51. Hofman, Z., R. Smeets, G. Verlaan, R. Lugt, and P. A. Verstappen. The effect of bovine colostrum supplementation on exercise performance in elite field hockey players. *Int. J. Sport Nutr. Exerc. Metab.* 12:461-469, 2002.

52. Hunter, A. Creatine and creatinine, monographs on biochemistry. New York, NY: Longmans, Green and Co, Ltd, 1928.

53. Hunter, D., K. Weintraub, G. Blackburn, and B. Bistrian. Branched chain amino acids as the protein component of parenteral nutrition in cancer cachexia. *Br. J. Surg.* 76:149-153, 1989.

54. Huq, F., M. Thompson, and P. Ruell. Changes in serum amino acid concentrations during prolonged endurance running. *Jpn. J. Physiol.* 43:797-807, 1993.

55. Jackson, A. S. and M. L. Pollock. Generalized equations for predicting body density in men. *Br. J. Nutr.* 40:497-504, 1978.

56. Jenkins, R. R. Symposium: oxidant stress, aging, and exercise. *Med. Sci. Sports Exerc.* 25:210-236, 1993.

57. Kashyap, S., E. Okamoto, S. Kanaya, C. Zucker, K. Abildskov, R. B. Dell, and W. C. Heird. Protein quality in feeding low birth weight infants: a comparison of whey-predominant versus casein-predominant formulas. *Pediatrics.* 79:748-755, 1987.

58. Keim, N. L., T. F. Barbieri, M. D. Van Loan, and B. L. Anderson. Energy expenditure and physical performance in overweight women: response to training with and without caloric restriction. *Metabolism.* 39:651-658, 1990.

59. Keys, A, J. Brozek, A. Henschel, O. Mickelsen, and H. L. Taylor. The biology of human starvation. University of Minnesota Press, Minneapolis MN, 1950.

60. Kimball, S. R. and L. S. Jefferson. Control of protein synthesis by amino acid availability. *Curr. Opin. Clin. Nutr. Meta. Care.* 5:63-67, 2002.

61. Kingsbury, K. J., L. Kay, and M. Hjelm. Contrasting plasma free amino acid patterns in elite athletes: association with fatigue and infection. *Br. J. Sports Med.* 32:25-33, 1998.

62. Kinscherf, R., V. Hack, T. Fischbach, B. Freidmann, C. Weiss, L. Edler, P. Bartsch, and W. Droge. Low plasma glutamine in combination with high glutamate levels indicate risk for loss of body cell mass in healthy individuals: the effect of N-acetyl-cysteine. *J. Mol. Med.* 74:393-400, 1996.

63. Kopple, J. D. Uses and limitations of the balance technique. *JPEN.* 11:79S-85S, 1987.

64. Koutedakis, Y., P. J. Pacy, R. M. Quevedo, D. J. Millward, R. Hesp, C. Borehamm, and N. C. C. Sharp. The effects of two different periods of weight-reduction on selected performance parameters in elite lightweight oarswomen. *Int. J. Sports Med.* 15:472-477, 1994.

65. Kramer, K., H. Dijkstra, and A. Bast. Control of physical exercise of rats in a swimming basin. *Physiol. Behav.* 53:271-276, 1993.

66. Lands, L. C., V. L. Grey, and A. A. Smountas. Effect of supplementation with a cysteine donor on muscular performance. *J. Appl. Physiol*. 87:1381-1385, 1999.

67. Lehmann, M., M. Huonker, F. Dimeo, N. Heinz, U. Gastmann, N. Treis, J. M. Steinacker, J. Keul, R. Kajewski, and D. Haussinger. Serum amino acid concentrations in nine athletes before and after the 1993 colmar ultra triathlon. *Int. J. Sports Med.* 16:155-159, 1995.

68. Lundsgaard, C., O. Hamberg, O. O. Thomsen, O. H. Nielsen, and H. Vilstrup. Increased hepatic urea synthesis in patients with active inflammatory bowel disease. *J. Hepatol.* 24:587-593, 1996.

69. Lunn, P. G., R. G. Whitehead, and B. A. Baker. The relative effect of a low protein-high carbohydrate diet on the free amino acid composition of liver and muscle. *Br. J. Nutr.* 36:219-230, 1976.

70. Maffulli, N. Making weight: a case study of two elite wrestlers. *Br. J. Sports Med.* 26:107-110, 1992.

71. Matthews, D. E. and R. G. Campbell. The effect of dietary protein intake on glutamine and glutamate nitrogen metabolism in humans. *Am. J. Clin. Nutr.* 55:963-970, 1992.

72. McArdle, A., D. Pattwell, A. Vasilaki, R. D. Griffiths, and M. J. Jackson. Contractile activity-induced oxidative stress: cellular origin and adaptive responses. *Am. J. Physiol. Cell. Physiol*. 280:C621-627, 2001.

73. McMurray, R. G., V. Ben-Ezra, W. A. Forsythe, and A. T. Smith. Responses of enduranced-trained subjects to caloric deficits induced by diet or exercise. *Med. Sci. Sports Exerc.* 17:574-579, 1985.

74. McMurray, R. G., C. R. Proctor, and W. L. Wilson. Effect of caloric deficit and dietary manipulation on aerobic and anaerobic exercise. *Int. J. Sports Med.* 12:167-172, 1991.

75. Medved, I., M. J. Brown, A. R. Bjorksten, and M. J. McKenna. Effects of intravenous N-acetylcysteine infusion on time to fatigue and potassium regulation during prolonged exercise. *J. Appl. Physiol.* 96:211-217, 2004.

76. Meisel, H. and R. J. Fitzgerald. Opioid peptides encrypted in intact milk protein sequences. *Br. J. Nutr.* 84(Suppl.1): S27-S31, 2000.

77. Micke, P., K. M. Beeh, and R. Buhl. Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients. *Eur. J. Nutr.* 41:12-18, 2002.

78. Miller, A. T. and C. S. Blyth. Estimation of lean body mass and body fat from basal oxygen consumption and creatinine excretion. *J. Appl. Physiol.* 5:73-78, 1952.

79. Mourier, A., A. X. Bigard, E. de Kerviler, B. Roger, H. Legrand, and C. Y. Guezennec. Combined effects of caloric restriction and branched-chain amino acid supplementation on body composition and exercise performance in elite wrestlers. *Int. J. Sports Med.* 18:47-55, 1997.

80. Myers, V. C. and M. J. Fine. The creatine content of muscle under normal conditions: its relation to the urinary creatinine. *J. Biol. Chem.* 14:9-26, 1913.

81. Neiman, D. C., K. A. Carlson, M. E. Brandstaer, R. T. Naegele, and J. W. Blankenship. Running endurance in 27-h-fasted humans. *J. Appl. Physiol.* 63:2502-2509, 1987.

82. Parry-Billings, M., E. Blomstrand, N. McAndrew, and E. A. Newsholme. A communicational link between skeletal muscle, brain, and cells of the immune system. *Int. J. Sports Med.* 11:S122-S128, 1990.

83. Parry-Billings, M., R. Budgett, Y. Koutedakis, E. Blomstrand, S. Brooks, C. Williams, P. C. Calder, S. Pilling, R. Baigrie, and E. A. Newsholme. Plasma amino acid concentrations in the overtraining syndrome: possible effects on the immune system. *Med. Sci. Sports Exerc.* 24:1353-1358, 1992.

84. Phillips, S. M., S. A. Atkinson, M. A. Tarnopolsky, and J. D. MacDougall. Gender differences in leucine kinetics and nitrogen balance in endurance athletes. *J. Appl. Physiol.* 75:2134-2141, 1993.

85. Phinney, S. D., E. S. Horton, E. A. Sims, J. S. Hanson, E. Danforth Jr., and B. M. LaGrange. Capacity for moderate exercise in obese subjects after adaptation to a hypocaloric, ketogenic diet. *J. Clin. Invest.* 66:1152-1161, 1980.

86. Platell, C., R. McCauley, R. McCulloch, and J. Hall. The influence of parenteral glutamine and branched-chain amino acids on total parenteral nutrition-induced atrophy of the gut. *JPEN.* 17:348-354, 1993.

87. Piatti, P. M., L. D. Monti, F. Magni, I. Fermo, L. Baruffaldi, R. Nasser, G. Santambrogio, M. C. Librenti, M. Galli-Kienle, A. E. Pontiroli, and G. Pozza. Hypocaloric high-protein diet improves glucose oxidation and spares lean body mass: comparison to hypocaloric high-carbohydrate diet. *Metabolism.* 43:1481-1487, 1994.

88. Rand, W. M., P. L. Pellett, V. R. Young. Meta-analysis of nitrogen balance studies for estimating protein requirements in healthy adults. *Am. J. Clin. Nutr.* 77:109-127, 2003.

89. Rankin, J. W., J. V. Ocel, and L. L. Craft. Effect of weight loss and refeeding diet composition on anaerobic performance in wrestlers. *Med. Sci. Sports Exerc.* 28:1292-1299, 1996.

90. Reid, M. B., D. S. Stokic, S. M. Koch, F. A. Khawli, and A. A. Leis. N-Acetylcysteine inhibits muscle fatigue in humans. *J. Clin. Invest.* 94:2468-2474, 1994.

91. Rigo, J., B. L. Salle, J. C. Picaud, G. Putet, and J. Senterre. Nutritional evaluation of protein hydrolysate formulas. *Eur. J. Clin. Nutr.* 49(Suppl. 1):S26-S38, 1995.

92. Rockwell, J., J. Walberg-Rankin, and B. Toderico. Creatine supplementation affects muscle creatine during energy restriction. *Med. Sci. Sport Exerc.* 33:621-68, 2001.

93. Roy, B. D., K. Luttmer, M. J. Bosman, and M. A. Tarnopolsky. The influence of post-exercise macronutrient intake on energy balance and protein metabolism in active females participating in endurance training. *Int J Sport Nutr Exerc Metab.* 12:172-188, 2002.

94. Rush, J. W. and S. D. Sandiford. Plasma glutathione peroxidase in healthy young adults: influence of gender and physical activity. *Clin. Biochem.* 36:345-351, 2003.

95. Sanchez, A. and M. E. Swendseid. Amino acid levels and enzyme activity in tissues of rats force-fed diets differing in methionine content. *J. Nutr.* 99:145, 1969.

96. Scholten, D. J., R. E. Morgan, A. T. Davis, and R. M. Albrecht. Failure of BCAA supplementation to promote nitrogen retention in injured patients. *J. Am. Coll. Nutr.* 9:101-106, 1990.

97. Scriver, C. R., D. M. Gregory, D. Sovetts, and G. Tissenbaum. Normal plasma free amino acid values in adults: the influence of some common physiological variables. *Metabolism.* 34:868-873, 1985.

98. Sen, C. K., M. Atalay, and O. Hanninen. Exercise-induced oxidative stress: glutathione supplementation and deficiency. *J. Appl. Physiol*. 77:2177-2187, 1994.

99. Shah, N. P. Effects of milk-derived bioactives: an overview. *Br. J. Nutr.* 84(Suppl.1):S3-S10, 2000.

100. Smith, A. T., D. R. Clemmons, L. E. Underwood, V. Ben-Ezra, and R. McMurray. The effect of exercise on plasma somatomedin-C/insulinlike growth factor I concentrations. *Metabolism.* 36:533-537, 1987.

101. Smith, R. and M. Elia. Branched-chain amino acids in catabolic states. *Proc. Nutr. Soc.* 42:473-487, 1983.

102. Smith, W. J., L. E. Underwood, and D. R. Clemmons. Effects of caloric or protein restriction on insulin-like growth factor-I (IGF-I) and IGF-binding proteins in children and adults. *J. Clin. Endocrinol. Metab.* 80:443-449, 1995.

103. Stanko, R. T., D. L. Tietze, and J. E. Arch. Body composition, nitrogen metabolism, and energy utilization with feeding of mildly restricted (4.2 MJ/d) and severely resticted (2.1 MJ/d) isonitrogenous diets. *Am. J. Clin. Nutr.* 56:636-640, 1992.

104. Stein, T. P., M. D. Schluter, M. J. Leskiw, and G. Boden. Attenuation of the protein wasting associated with bed rest by branched-chain amino acids. *Nutrition*. 15:656-660, 1999.

105. Tipton, K. D., A. A. Ferrando, S. M. Phillips, D. Doyle Jr., and R. R. Wolfe. Postexercise net protein synthesis in human muscle from orally administered amino acids. *Am. J. Physiol.* 276:E628-634, 1999.

106. Van Loan, M. D., N. L. Keim, T. F. Barbieri, and P. L. Mayclin. The effects of endurance exercise with and without a reduction of energy intake on fat-free mass and the composition of fat-free mass in obese women. *Eur. J. Clin. Nutr.* 48:408-415, 1994.

107. Vaswani, A. N., D. Vartsky, K. J. Ellis, S. Yasumura, and S. H. Cohn. Effects of caloric restriction on body composition and total body nitrogen as measured by neutron activation. *Metabolism.* 32:185-188, 1983.

108. Velthuis-te Wierik, E. J. M., L. V. Hoogzaad, H. van den Berg, and G. Schaafsma. Effects of moderate energy restriction on physical performance and substrate utilization in non-obese men. *Int. J. Sports Med.* 15:478-484, 1994.

109. Viru, A. Mobilisation of structural proteins during exercise. *Sports Med.* 4:95-128, 1987.

110. Walberg, J. L., M. K. Leidy, D. J. Sturgill, D. E. Hinkle, S. J. Ritchey, and D. R. Sebolt. Macronutrient content of a hypoenergy diet affects nitrogen retention and muscle function in weight lifters. *Int. J. Sports. Med*. 9:261-266, 1988.

111. Walberg, J. L., V. K. Ruiz, S. L. Tarlton, D. E. Hinkle, and F. W. Thye. Exercise capacity and nitrogen loss during a high or low carbohydrate diet. *Med. Sci. Sports Exer.* 20:34-43, 1988.

112. Walberg-Rankin, J. Changing body weight and composition in athletes. in Perspectives in Exercise and Sports Medicine vol 11 : Exercise, Nutrition, and Control of Body Weight. eds. D. Lamb and R. Murray, Cooper Publ., Carmel IN, pp 199-242, 1998.

113. Walberg-Rankin, J., C. E. Hawkins, D. S. Fild, and D. R. Sebolt. The effect of oral arginine during energy restriction in male weight trainers. *J. Strength Cond. Res,* 8:170-177, 1994.

114. Webster, S., R. Rutt, and A. Welstman. Physiological effects of a weight loss regimen practiced by college wrestlers. *Med. Sci. Sports Exerc.* 22:229-234, 1990.

115. Widerman, P. M. and R. D.Hagan. Body weight loss in a wrestler preparing for competition: a case report. *Med. Sci. Sports Exerc.* 14:413-418, 1982.

116. Wong, C. W. and D. L. Watson. Immunomodulatory effects of dietary whey proteins in mice. *J. Dairy Res.* 62:359-368, 1995.

117. Zachwieja, J. J., D. M. Ezell, A. D. Cline, J. C. Ricketts, P. C. Vicknair, S. M. Schorle, and D. H. Ryan. Short-term dietary energy restriction reduces lean body mass but not performance in physically active men and women. *Int. J. Sports Med.* 22:310-316, 2001.

118. Zhang, P. C. and C. P. Pang. Plasma amino acid patterns in cancer. *Clin. Chem.* 38:1198-1199, 1992.

Vita

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