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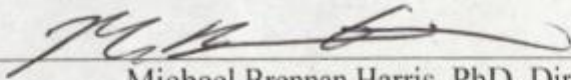
**The Efficacy and Safety of Six-Weeks of Pre-Workout Supplementation in Resistance Trained Rats**

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelors of Science in Inderdisciplinary Studies: Biological Chemistry from The College of William & Mary

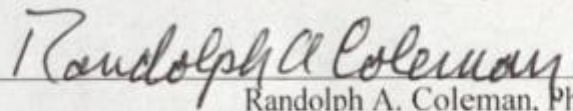
By

Justin Paul Canakis

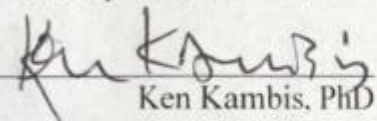
Accepted for Honors \_\_\_\_\_



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April 21, 2017

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## **Abstract**

Pre-workout supplements (PWSs) contain a cocktail of ingredients that are marketed to increase energy levels, endurance, and muscle power. PWSs are not regulated by the Food and Drug Administration (FDA) and have a history of causing adverse side effects. The goal of this study is to independently analyze the efficacy and safety of Pro Supps Mr. Hyde (MH) in resistance trained rats. Data suggest that six weeks of pre-workout supplementation in rats undergoing resistance training results in modest efficacy with improvement in amount lifted but no change in muscle mass. Urinary creatine tests and vascular reactivity tests were selected as biological markers of safety. Urinary creatinine tests revealed that the MH group demonstrated elevated creatinine levels; however, urinary creatinine may not be a conclusive indicator in determining renal function. Data from vascular functions tests revealed no significant differences. More research needs to be conducted because there is a scarcity of literature that explores the effects of PWSs on resistance training and safety. Given that each formula has a unique proprietary blend, a general consensus regarding the efficacy and safety of PWSs cannot be inferred. This study is novel because it is the first of its kind that conducts an independent analysis of acute MH supplementation. Future research efforts are still necessary due to the popularity of PWSs, the lack of regulation and accountability, and the high degree of product variability.

## **Acknowledgement**

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*Justin Paul Canakis*

## Background

### *DSHEA and Its Effects on the VMS Industry.*

In 1994, the Dietary Supplement Health and Education Act (DSHEA) was enacted. DSHEA redefined the meaning of dietary supplements and consequently created a highly lucrative market with loose regulation standards. Prior to DSHEA, dietary supplements were tightly regulated by the Food and Drug Administration (FDA) and supplements were limited to vitamins, minerals, and proteins. In the post-DSHEA era, dietary supplements were redefined to include “a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total dietary intake or a concentration, metabolite, constituent, extract, or combination of any ingredient described in the clause” (US Food and Drug Administration, 1994. sec. 3). Additionally, DSHEA declared that dietary supplements were exempt from pre-market FDA approval. Proponents of DSHEA and its pre-market FDA approval rule claim there are “comprehensive requirements in place for advertising claims that ensure that consumers have access to truthful and non-misleading information,” and that the FDA has the authority to remove unsafe products and cease deceptive advertising (McCormick, 2014. sec. 5). In reality, however, the passage of DSHEA has been primarily advantageous for the Vitamins, Minerals, and Supplements (VMS) industry because products do not need to go through the expensive and time consuming regulatory hurdles of pre-market approval. According to Forbes, it is projected that the VMS industry will be valued around \$60 billion by 2021 (Lariviere, 2015). With so much money at stake, there has been fierce competition amongst companies to formulate propriety blends—without undergoing scientific scrutiny—that offer the consumer more intense and efficacious workouts.



*History of Adverse Side Effects from Pre-Workout Supplements.*

The PWS industry has taken advantage of the loose regulations established by DSHEA, as is evident through its history of mislabeling products, including banned substances in proprietary blends, and causing adverse side effects in consumers. For example, the PWS JACK3D (pronounced “jacked”) made the news after it caused the death of Michael Lee Sparling, a 22-year-old Army private, on March 15, 2013 (Singer & Lattman, 2013). Private Sparling consumed JACK3D before he went on a run with his unit, and subsequently went into cardiac arrest and died. The potent ingredient in JACK3D that is believed to cause Private Sparling’s death was 1-3dimethylamylamine (DMAA). DMAA is structurally similar to an amphetamine and elevates blood pressure. On April 11, 2013, the FDA issued a warning against the use of DMAA. However, the FDA had enough evidence to issue a statement much earlier—which could have saved Private Sparling’s life. For example, the FDA received 86 reports from 2007-2013 linking DMAA use to seizures, arrhythmias, heart attacks, strokes, panic attacks, and deaths, along with exacerbation of psychiatric conditions (US Food and Drug Administration, 2013). Another example of the FDA’s lack of oversight in the VMS industry is the discovery of methamphetamine analog—N,  $\alpha$ -diethyl-phenylethylamine (N,  $\alpha$ -DEPEA)—that was not listed on the ingredients label of a PWS called Craze. Cohen, Travis, and Venhuis (2014) described the compound as a potentially dangerous designer drug with entirely unknown physiological consequences. Given the popularity of PWSs and the industry’s lack of accountability, more studies need to be conducted to determine the efficacy and safety of the proprietary blends in PWSs.

## **Ingredient Analysis**

Figure 1 depicts the supplement facts that are listed on the container of Pro Supps Mr. Hyde (MH). MH is a unique proprietary blend that contains three matrices: a strength matrix, a caffeine matrix, and an intensity matrix. The strength matrix (5g) is composed of 2.5g beta alanine, 1g creatine nitrate, 500mg L-leucine, 500mg agmatine sulfate, and 500mg L-citrulline aspartate. The caffeine matrix (419mg) is composed of 300mg caffeine anhydrous, 69mg dicationic caffeine malate, and 50mg caffeine citrate. The intensity matrix (154mg) is composed of 50mg hordenine, 50mg picamilon, 50mg N-methyl L-tyramine HCl, 2mg yohimbe bark extract, and 2mg rauwolfia vomitoria root extract. The following subsections will discuss the metabolic and physiological effects of each ingredient in more detail.

### *Beta-Alanine (2.5g).*

Beta-alanine is a naturally occurring amino acid that is hypothesized to enhance cardiovascular exercise performance and improve muscular endurance. The highest concentrations of beta-alanine are found in skeletal muscle; thus the main dietary source of beta-alanine is meat. For example, one gram of beef contains approximately 1853 $\mu$ g of beta-alanine, and one gram of chicken contains approximately 666 $\mu$ g of beta-alanine (Gil-Agustí, Esteve-Romero, & Carda-Broch, 2008). The recommended dietary dosage of beta-alanine is 2-5g, which is readily achieved through a well-balanced diet. Upon digestion, beta-alanine is metabolized into its active metabolite, carnosine (beta-alanyl-L-histidine), via the ATPGD1 enzyme (carnosine synthase). In response to drops in pH, carnosine is released and hydrolyzed into beta-alanine and histidine via carnosinase-1 and carnosinase-2 (CN1 and CN2). The histidine residues from carnosine hydrolysis act as proton buffers and normalize pH levels. Beta-alanine is the rate-

limiting factor in this metabolic pathway and is an attractive workout supplement because it can reduce acidosis without influencing oxygen uptake (Baguet, Koppo, Pottier, & Derave, 2010).

Beta-alanine is a popular ingredient in pre-workout supplements because it is relatively safe to consume. After reviewing the mechanisms of beta-alanine metabolism, it is feasible that beta-alanine supplementation can improve muscular endurance and hypertrophy. The main side effect of excessive beta-alanine consumption is called paresthesia, a harmless tingling; aside from paresthesia, there are no other reported side-effects. Oddly enough, some bodybuilders intentionally induce paresthesia on themselves because they enjoy the feeling before they workout; however, beta-alanine supplementation is not time dependent. Kern and Robinson (2011) conducted a study that explored the effects of beta-alanine supplementation on performance and body composition in collegiate wrestlers and footballs players. In a double-blind, placebo-controlled study, subjects (n=37) ingested either 4g of beta-alanine (BA) or placebo (PL) for eight weeks. Subjects performed preliminary and posttreatment trials of a 300-yard shuffle and a 90-degree flexed-arm hang (FAH). Data suggested a trend in which the BA group outperformed the PL group. Interestingly, the researchers found that the BA group displayed increases in lean mass whereas the placebo group displayed losses in lean mass. When viewed holistically, this study supports the idea that beta-alanine may improve both performance and muscle hypertrophy in trained athletes. A major flaw in the study is that it lacks external validity because the subjects were collegiate athletes and do not represent the population as a whole.

Walter, Smith, Kendal, Stout, and Cramer (2010) performed a double-blind randomized study that explored the effects of six weeks of high-intensity interval training with and without

beta-alanine supplementation in women. Subjects (n=44) were divided into three groups: a beta-alanine (BA) group (n=14) that consumed 1.5 g beta-alanine + 15 g dextrose; a placebo (PL) group (n=19) that consumed 16.5 g dextrose; and a control (CTRL) group (n=11) that consumed nothing. Subjects performed five sets of high-intensity interval training on a bike 3 times per week. The researchers tested maximal oxygen consumption rate, ventilatory threshold, and body composition at week 0, week 4, and week 8. At the end of the study, the BA group experienced an increases in body mass and decreases in fat percentage.

Stout et al. (2008) conducted a double-blind, randomized study in an elderly population (55-92 year) and explored the effect of 90 days of beta-alanine supplementation on neuromuscular fatigue. This study is notable because the aging population is generally deficient in skeletal muscle concentration of carnosine and is consequently less efficient at buffering exercise-induced lactic acid. Subjects (n=26) were divided into two groups—800 mg beta-alanine x 3 per day (n=12) or placebo (n=14)—and performed pre and post trials of cycling. Researchers measured the physical working capacity at the fatigue threshold in the elderly subjects and found significant improvement in the beta-alanine group. This study explored the effects of a carnosine deficient population and confirmed that supplementation works best when there is a deficiency. Taken together, the three studies described above work synergistically and support the hypothesis that beta-alanine can improve muscular endurance and muscular hypertrophy in a wide-variety of populations.

*Creatine Nitrate (1g).*

Creatine is a naturally occurring compound that has important functions in the brain and skeletal muscle. It is hypothesized that creatine can improve muscular strength and power output by increasing cellular reserves of ATP. Natural sources of creatine include beef (5g creatine/1.1kg) and chicken (3.4g creatine/1kg) (Harris, Lowe, Warnes, & Orme, 1997). The recommended dosage for creatine is 2g/day; however, the average daily consumption of creatine for men and women is 1.08g and .64g, respectively. Thus, creatine supplementation may be advantageous because the population is deficient—and supplementation works best when correcting deficiencies. Creatine is a small peptide composed of L-arginine, glycine, and methionine. Creatine is primarily synthesized in the liver; in the first step of creatine synthesis, glycine and arginine are converted into ornithine and guanidoacetate via arginine:glycine aminotransferase (AGAT). Next, guanidoacetate receives a methyl group from S-adenosylmethionine (SAM) via guanidinoacetate methyltransferase and forms the products creatine and adenosylhomocysteine. SAM is a major methyl donor in the human body that is involved in a number of metabolic processes such as epigenetic modulation, maintaining cell membranes, and producing and breaking down neurotransmitters (Ehrlich, 2015). Interestingly, creatine metabolism accounts for 40% of the SAM used in the body for methylation processes. Thus, creatine supplementation may attenuate the metabolic burden of creatine synthesis and serve a protective role.

Creatine is an energy substrate that stores high energy phosphate groups in the form of phosphocreatine and can increase rates of cytoplasmic ATP regeneration. Creatine is an attractive supplement because it can theoretically ameliorate the exchange of ADP into ATP and

consequently improve strength and increase muscle mass. The specific form of creatine used in the strength matrix of MH is creatine nitrate. Creatine nitrate is more soluble and palatable than other forms of creatine; however, there is not a sufficient amount of scientific evidence that supports its use over other sources of creatine, such as creatine monohydrate.

A study conducted by Joy et al. (2014) explored the safety of 28 days of creatine nitrate (CrN) supplementation in healthy individuals. Subjects (n=58) were randomly divided in to three groups: 1 g/day of CrN (n=18), 2g/day of CrN (n=20), or control group (n=20). The researchers measured red blood cell distribution width, platelets, absolute monocytes, creatinine, blood urea nitrogen (BUN) creatinine, sodium protein, and alanine aminotransferase (ALT) as biological safety markers. The researchers found that BUN and BUN:creatinine increased beyond the clinical reference range for the 2 g treatment, but did not reach statistical significance (Joe et al., 2014). The researchers concluded the increased levels had minor clinical concern and that CrN supplementation has no adverse side effects in daily doses up to 2g over 28 days. Mr. Hyde contains 1g of creatine nitrate in its formula, and is in line with the ‘safe’ dosages reported in the literature. However, it is unknown how creatine nitrate compounds with the ten other supplements listed on Mr. Hyde’s label.

Galvan et al. (2016) performed a study to determine the safety and exercise performance-characteristics of creatine nitrate (CrN) supplementation relative to creatine monohydrate (CrM) supplementation. In a randomized, double-blind study, subjects (n=48) were divided into a 1.5g CrN-low group, a 3g CrN-high group and a 3g CrM group. The 28-day trial consisted of a 7-day interim testing period followed by loading sequences (4servings/day). On day 7 and day 28, subjects performed bench presses, Wingate testing, and a cycling sprint. The researchers found

no differences in performance amongst the groups; interestingly, all groups demonstrated significant increases in bench press lifting volume, bench press peak power, and average power. The researchers concluded that creatine nitrate has no safety concerns and its physiologic effects comparable to creatine monohydrate.

In light of the scarcity of scientific evidence regarding creatine nitrate, this paper utilizes the comparable safety and efficacy characteristics between creatine nitrate and creatine monohydrate to explore the effects of creatine supplementation on muscle hypertrophy, renal function, and vascular function. Rawson and Volex (2003) performed a meta-analysis of 22 studies and explored the effects of creatine supplementation and resistance training on muscle strength and weightlifting performance. The researchers found an 8% increase in muscle strength following creatine supplementation plus resistance training. Furthermore, the researchers found a 14% increase in weightlifting performance. For example, increases in bench press performance ranged from 16-43% following creatine supplementation. The underlying mechanisms of creatine metabolism and its corresponding physiologic effects support the hypothesis that creatine supplementation, in combination with resistance training, improves performance and muscle hypertrophy.

A major safety concern hypothesized when conducting The Efficacy and Safety of Pre-Workout Supplements in Resistance Trained Rats was that creatine may induce strain on the kidneys because creatinine is a by-product of creatine metabolism that is excreted in urine. The best study that refutes this hypothesis comes from a case study on an individual with a single functioning kidney. The study explored the effect of short-term, high-dose creatine supplementation on measured glomerular filtration rate (GFR). The 20-year old man took

creatine for 35 days—20g/day for the first 5 days followed by 5 g/day for the next 30 days. Gualano, Ferreira, Sapienza, Segura, and Lancha (2010) measured Cr-EDTA clearance, proteinuria, electrolyte levels, albuminuria, serum urea level, and creatinine clearance. Data from these multiple tests suggests that short-term creatine supplementation does not affect kidney function. Interestingly, the researchers reported an increase in serum creatinine levels, which parallels the increase in urinary creatinine levels found in The Efficacy and Safety of Pre-Workout Supplements in Resistance Trained Rats. However, in light of the multiple biological markers used to measure renal health, the increase in creatinine levels cannot be used as a conclusive biological marker.

The relationship between creatine and vascular function is not well-defined. In fact, the effects of creatine supplementation on systemic microvascular reactivity and density were never reported until de Moraes R.D., Van Bavel, de Moraes B.S., and Tibiriçá (2014) conducted an open-label study on 40 subjects that received 20g/day of creatine monohydrate for one week. Data from laser speckle contrast imaging and intra-vital video microscopy revealed that creatine supplementation led to an increase—but not a significant increase—in cutaneous microvascular vasodilation (de Moraes R.D. et al., 2014). For the first time, the researchers discovered that creatine can improve systemic endothelial-dependent microvascular reactivity in healthy, young adults. Due to the open-label design of the study, the internal validity of the study may be compromised. However, this novel study may serve as a catalyst to further explore and elucidate the effects of creatine on vascular function.



*L-Leucine (500 mg).*

Leucine (2-amino-4-methylpentanoic acid) is one of three branch chain amino acids (BCAAs) and is often referred to as the 'main' BCAA because it is a strong activator of mTORc1 and is exclusively ketogenic. Leucine has a high affinity for mammalian Target of Rapamycin complex one (mTORc1). It is hypothesized that leucine leads to an influx of intracellular calcium and subsequently activates mTORc1. Upon mTORc1 activation, a signaling cascade induces muscle protein synthesis via p70S6K. Leucine is an attractive compound to include in pre-workout supplements because it has anabolic properties in skeletal muscle. It may be beneficial to preload leucine before a workout because the anabolic effects of leucine are augmented by physical exercise. For example, Tipton et al. (2001) conducted a study that explored the relationship between the timing of essential amino acid-carbohydrate (EAC) ingestion and the anabolic response of muscle to resistance exercise. The EAC solution contained .65mg histidine, .60mg isoleucine, 1.12mg leucine, .95mg lysine, .19mg methionine, .93mg phenylalanine, .88mg threonine, and .7mg valine. Healthy human subjects (n=6) participated in two trials in random order in which they consumed EAC before or after resistance training. The resistance training consisted of 10 sets of 8 repetitions of leg press at 80% of 1RM and 8 sets of 8 repetitions of leg extension at 80% 1RM. The results indicated that loading before resistance exercise increased net muscle protein synthesis. A limitation of this study is that it did not examine the independent effects of leucine; however, there are no studies that explore this phenomenon. Thus, we cannot infer that supplementing leucine before a workout is more beneficial, but these findings remain consistent with the protein synthesis stimulating properties of leucine.

Ispoglou, King, Polman, and Zanker (2011) investigated the effects of daily leucine supplementation in novice trainees during a 12-week weight training program. In a double-blind trial, untrained subjects (n=26) ingested either 4 g/d of leucine or a lactose placebo and performed a prescribed resistance training program using eight standard exercise machines. The researchers quantified strength via a five repetition maximum (5-RM) and found that the leucine group performed significantly better at the 5-RM strength test. Thus, on a mechanistic and experimental level, leucine supplementation may be a beneficial pre-workout supplement due to its high affinity for mTORc1 and its ability to enhance strength performance.

The ideal amount of leucine has not been established by the scientific community, but the average dose of leucine in most scientific studies is approximately 2.5 g. The lack of guidelines regarding recommended leucine consumption may lead to excess leucine intake. Some side effects of excessive leucine intake include ammonia buildup, pellagra, hypoglycemia, and gastrointestinal distress (King, 2016). Elango, Chapman, Rafii, Ball, and Pencharz (2012) conducted a study to determine the tolerable upper intake level of leucine in acute dietary studies in young men (n=5). The subjects were given a graded leucine intake up to 1,250mg/kg, which is 25-fold the estimated average requirement. The researchers found that oral doses of 500-1,250 mg/kg led to increased serum ammonia. Increased serum ammonia can be toxic to the liver and kidneys because they aid in filtering out excess ammonia from the blood stream. Thus, the researchers established an upper limit of 500mg/kg for a 150lb human. Regardless of the small sample size of this study, it serves a novel purpose in establishing a limit for leucine consumption. While the general population may not be at risk for leucine toxicity, this study is especially relevant for bodybuilders who are prone to consuming nutritional supplements in order to give them a competitive edge. Based on these studies, the 500mg of leucine contained in

Mr. Hyde does not appear to be an independent risk factor for leucine toxicity in rats; however, it may be a contributing factor when used in combination with other compounds.

*Agmatine Sulfate (500mg).*

Agmatine is a metabolite of L-arginine that is derived through a decarboxylation reaction. Agmatine sulfate (1-Amino-4-guanidinobutane sulfate salt) is simply the salt form of agmatine that can be added to formulas, such as MH. Agmatine is considered to be a neuromodulator with a variety of functions. For example, agmatine is an antagonist for N-methyl-D-aspartate (NMDA), nicotinic acetylcholine receptor (nAChR), nitric oxide synthase enzymes, calcium channels, and certain serotonin receptors. Oddly, agmatine supplementation can increase the perception of pain, but works in a synergistic fashion with opioids to reduce pain killer tolerance.

Agmatine, the active ingredient in agmatine sulfate, is naturally found in fermented foods such as wine and beer—but these concentrations are not biologically relevant. The recommended dosage of agmatine is 2,670mg; however, this statistic is from one of the few studies that test agmatine on human subjects (Keynan, Mirovsky, Dekel, Gilad, V. H., & Gilad, G. M., 2010). The lack of human studies on agmatine raises serious red flags; what is even more worrisome about the addition of agmatine in MH is that animal studies have only explored intravenous injects. The oral ingestion of agmatine may be biologically irrelevant because agmatine is poorly absorbed due to its properties as basic biogenic amine with a high degree of hydrogen bonding and lipophilicity (Remko, Swart, & Bickelhaupt, 2006). Furthermore, when agmatine is consumed with protein, it is competitively inhibited by L-arginine because both compounds utilize the same receptors for biological uptake. Thus, the inclusion of agmatine sulfate in Mr. Hyde may have no biological relevance to the consumer, but for the sake of consistency this

paper will still mention agmatine metabolism and its effects on muscle hypertrophy, vascular function, and renal function.

The pathway of agmatine metabolism is not fully understood; it is hypothesized that agmatine is converted to 4-guanidinobutyrate via the diamine oxidase (DAO) enzyme. DAO is highly expressed in gastrointestinal and epithelial tissue, smooth muscle cells of the endothelium, and the kidneys. Holt and Baker (1995) found that that agmatine is metabolized in the kidneys and enhances GFR in kidney tissues, but the mechanism is not fully understood.

There is a paucity of research exploring the relationship between agmatine and muscular performance. Hwang, Liu, Tzeng, and Cheng (2005) conducted a study that investigated the effect of agmatine on plasma glucose in diabetic rats. The researchers measured mRNA and protein levels of glucose transporter subtype 4 (GLUT4) in the soleus muscle via northern blotting and western blotting, respectively. The researchers found that agmatine decreased plasma glucose in a dose-dependent manner and concluded that agmatine may activate imidazole receptors in the adrenal gland, and subsequently enhance GLUT4 gene expression and lower plasma glucose levels. In theory, agmatine can increase skeletal muscle glucose uptake and improve muscular metabolism; however, the validity of this claim is yet to be replicated, and agmatine most likely has no relevant effect on muscle hypertrophy.

Regarding vascular function, agmatine possess both positive and negative regulatory mechanisms. Agmatine has a high affinity to bind to the  $\alpha_2A$  receptor and can induce nitric oxide synthesis and act as a vasodilator. Relative to other  $\alpha_2A$  receptor agonists, such as L-arginine and L-citrulline, agmatine is 100-fold more potent in activating the receptor (Joshi et al., 2007). Interestingly, it has also been demonstrated that agmatine can inactivate nitric-oxide

synthase (NOS) enzymes—this property may explain why agmatine can be used to prevent opioid tolerance (Demady et al., 2001). For example, agmatine causes a 3-fold increase in the NADPH oxidase activity of nNOS and leads to an inactivation of the enzyme (Demady et al., 2001). Thus, the dual ability of agmatine to serve as a vasodilator and inhibit nNOS has perplexed researchers and the mechanisms are still poorly understood.

There is not a conclusive amount of evidence to make a definitive statement regarding the safety and efficacy of agmatine for use in pre-workout supplements. There has been one human trial that explored the safety and efficacy of dietary agmatine sulfate in lumbar-disc associated radiculopathy. Participants were divided into two groups: an agmatine sulfate group or a placebo group. The agmatine sulfate group consumed a 2.670 g/day for 14 days and the researchers concluded that dietary agmatine sulfate is a safe and efficacious treatment for alleviating pain and improving quality of life in lumbar disc-associated radiculopathy. Despite the conclusions of this study—which is the only study on agmatine that utilizes human subjects—there needs to be more research in order to determine the short term and long term effects of supplementation. It is evident that the physiological effects of this compound are far from being elucidated, and brings into question why it is included in the Mr. Hyde formula. It is irresponsible to include an ingredient with such little mechanistic and pharmacological knowledge in a formula marketed to healthy adults. There is a dearth of evidence that explores the independent role of agmatine sulfate in the human body, and even less evidence exploring the compounding effects of supplementing agmatine with other ingredients included in PWSs.

*Citrulline:Aspartate 1:1 (500mg).*

L-citrulline is an amino acid that is metabolized to L-arginine in the kidneys. L-citrulline is included in MH because the underlying mechanisms of L-citrulline metabolism suggest that supplementation can reduce fatigue and improve performance. The recommended dosage of dietary L-citrulline ranges from 1,000-3,000 mg. Natural sources include watermelon, pumpkins, squash, and cucumbers; however, L-citrulline is neither an essential amino acid nor a common dietary amino acid. The body can produce L-citrulline from two different pathways. The major pathway that accounts for 90% of circulating L-citrulline is the plasma glutamine pathway (Curis et al., 2005). In the plasma glutamine pathway, the enzyme ornithine transcarbamylase uses ornithine and carbamoyl phosphate (which requires glutamine) to produce L-citrulline. The arginine pathway—the minor pathway—accounts for 10% of circulating L-citrulline. In the arginine pathway, L-citrulline can be produced directly or indirectly. The direct method occurs in the nitric oxide cycle when arginine loses a nitric oxide molecule via nitric oxide synthase and is converted into L-citrulline. The indirect method occurs in the urea cycle in which arginine is converted into ornithine via arginase; next, ornithine and ammonia are catalyzed into L-citrulline via carbomyl phosphate. L-citrulline is one of three dietary amino acids that is involved in the urea cycle along with L-arginine and L-ornithine. Thus, L-citrulline supplementation can increase biological reserves of arginine and ornithine, ameliorate the ammonia recycling process, and improve nitric oxide metabolism. Interestingly, L-citrulline supplementation is more efficient in increasing plasma arginine levels than arginine supplementation itself. For example, researchers reported that 5-10g L-citrulline supplementation can double or triple ornithine and arginine levels while L-citrulline follows a linear dose-response pattern (Moinard et al., 2008).

One potential reason for this phenomena is that L-citrulline is better absorbed in the intestines relative to arginine (Curis, Crenn, & Cynober, 2007).

In skeletal muscle, L-citrulline has been reported to improve performance by increasing ATP efficiency. Animal models reported that citrulline may modulate enzymes in the glycogenolysis and glycolysis pathways and subsequently induce a shift from aerobic metabolism to anaerobic metabolism (Faure et al., 2013). In humans, L-citrulline may benefit weightlifting secondary to reducing fatigue. Pérez-Guisado and Jakeman (2010) performed a randomized, double-blind, 2-period crossover design study in which subjects (n=41) were given 8 g of L-citrulline malate or placebo and performed a bench press. The researchers reported that the L-citrulline malate group demonstrated a significant increase in number of repetitions and a significant decrease in pectoral muscle soreness at 24 hours and 48 hours. The only reported side effect was gastrointestinal distress, which is expected from such a high dose of L-citrulline. Hence, the researchers concluded that L-citrulline may be helpful in increasing performance in high-intensity anaerobic exercises.

Ochiai et al. (2012) conducted a study that explored the relationship between L-citrulline, vascular function, and arterial stiffness in middle aged men. In a double-blind, randomized, placebo-controlled, parallel-group trial, subjects (n=15) with a mean age of 58 years were given 5.6g/day of L-citrulline (n=8) or placebo (n=7) for seven days. The researchers quantified arterial stiffness via brachial-ankle pulse wave velocity (baPWV). Data revealed a significant reduction in baPWV, significant increases in serum nitric oxide, and significant increases plasma L-citrulline and arginine concentrations. The researchers concluded that short-term L-citrulline supplementation may improve arterial stiffness, independent of blood pressure in humans.

The inclusion of L-Citrulline:Aspartate 1:1 (500mg) in MH is based on legitimate science. On a metabolic level, L-citrulline may improve renal function due to its role in the urea cycle. Additionally, L-citrulline supplementation may benefit vascular function due to its ability to improve arterial stiffness independent of blood pressure. Regarding muscle hypertrophy, L-citrulline may be beneficial for athletes performing high intensity anaerobic exercises. Hence, acute citrulline supplementation is an attractive target for pre-workout formulas and appears to be safe and efficacious.

*Caffeine Matrix (419mg): Caffeine Anhydrous (300mg), Dicafeine Malate (69mg), Caffeine Citrate (50mg).*

Caffeine (1,3,7-trimethylxanthine) is a popular drug because it is a powerful stimulant and a systemic vasoconstrictor that provides mental stimulation and improves strength and endurance. Caffeine is naturally found in coffee (40-180mg / 150ml), tea (24-50mg / 150ml), soda (15-29mg/ 180ml), and chocolates (Knight, C. A., Knight, I., & Mitchell 2006). The recommended single dose of caffeine is 200mg (~3mg/kg) and the threshold for daily caffeine intake is 400mg—MH contains 419mg of caffeine and violates both recommendations (Tetens, 2015). Caffeine is highly absorbed in the gut; upon ingestion, approximately 84% of caffeine is demethylated into paraxanthine via the enzymes CYP1A1/2 (Caubet, Elbast, Dubuc, & Brazier, 2002). Variations in CYP2A1/2, either genetic or through supplements, can greatly affect the pharmacokinetics and cause unique physiologic responses. Thus, MH supplementation may be particularly dangerous because it contains dangerously high levels of caffeine in a single dose.

Caffeine is one of the most well-studied supplements and has been demonstrated to improve exercise performance. Smirmaul, de Moraes, Angius, and Marcora (2017) investigated



the effects of caffeine on performance, neuromuscular fatigue, and perception of effort during high-intensity cycling exercise in hypoxic conditions. In a double-blind, randomized, counterbalanced, cross-over design, subjects (n=7) performed a time to exhaustion test on a cycle ergometer under the influence of 4 mg/kg of caffeine or placebo. The researchers found that caffeine significantly improved time to exhaustion by 12% and significantly decreased subjective fatigue (Smirmaul et al., 2017). This study is just one example of the beneficial effects of caffeine on improving exercise performance. Thus, it is no wonder why virtually every pre-workout supplement and energy drink contains caffeine.

Moderate levels of caffeine have been demonstrated to significantly decrease risks of heart attack (Mostofsky, Rice, Levitan, & Mittleman, 2012). However, the high levels of caffeine that are contained in PWSs and energy drinks may be detrimental with regards to cardiac and vascular health. Lippi, Cervellin, and Sanchis-Gomar (2016) examined the relationship between these caffeine-rich supplements and myocardial ischemia. The researchers found 8 case reports that linked energy drinks to myocardial ischemia in which there were no additional triggers of heart attack besides the intake of excess caffeine. Unsurprisingly, they found that the caffeine-rich supplements (80-160 mg of caffeine per can) increased heart rate, cardiac outputs and contractility, stroke volume, and arterial blood pressure (Lippi et al., 2016). Furthermore, they found 'biological abnormalities' such as increased platelet aggregation, endothelial dysfunction, hyperglycemia, and increased total cholesterol. Although the correlations made in this study cannot infer causation, it should serve as warning sign for consumers (and the FDA) that are ingesting high, single doses of caffeine from multiple synthetic forms.

The caffeine matrix in MH contains 419mg of caffeine from three different sources—300mg caffeine anhydrous, 69mg dicaffeine malate, and 50mg caffeine citrate. After analyzing the caffeine content of 49 popular pre-workout products, Mr. Hyde was found to contain the highest levels of caffeine content with 419 mg/scoop. The range of caffeine was 60-419 mg/scoop, the mean amount of caffeine was 207 mg/scoop, and the median and mode were 200 mg/scoop. These findings are especially alarming because the caffeine content in Mr. Hyde is drastically high relative to other pre-workout supplements.

MH contains three different forms of caffeine that may have dangerous health consequences when acting synergistically. The first form of caffeine listed on the ingredients lists is caffeine anhydrous. Caffeine anhydrous is the typical caffeine source found in most products due to its low cost, high stability, and decent solubility. Caffeine anhydrous exists in capsules, tablets, powders, and liquid forms. Upon ingestion, caffeine anhydrous can increase caffeine blood levels as quickly as 15-45 min with levels peaking 60-120 min post-ingestion (Goldstein et al., 2010). Dicaffeine malate is the next form of caffeine listed in the caffeine matrix in MH. Dicaffeine malate, also referred to as 'infinergy', is an ionically bonded combination of caffeine and malic acid. It has been reported that malic acid can calm the digestive distress induced by natural caffeine and caffeine anhydrous (Acheson, Zahorska-Markiewicz, Pittet, Anantharaman, & Jéquier, 1980). Interestingly, after cocaine was banned from use in consumer products, Coca-Cola used dicaffeine malate as a replacement stimulant. Thus, dicaffeine malate may serve as a more potent form of caffeine that can increase focus, energy, metabolism, and decreased perception of fatigue. The third form of caffeine in MH is caffeine citrate; caffeine citrate is a synthetic combination of caffeine anhydrous, citric acid monohydrate, and sodium citrate dehydrate (RxList, 2017). Caffeine citrate is new to the

bodybuilding community, but has been traditionally prescribed in the clinical setting to treat apnea of prematurity and migraines because it is rapidly absorbed. For example, it has been reported that caffeine blood levels can peak as quickly as 30min post-ingestion (Charles, Townsend, Steer, Flenady, Gray, & Shearman 2008). The multiple synthetic forms of caffeine and unparalleled concentration of caffeine contained in MH pose a serious public health risk to consumers. Consumers need to be cautious and avoid consuming coffee, tea, soda, or energy drinks the same day they take pre-workout supplements because they may surpass the threshold for daily caffeine intake (400mg).

*Hordenine (50mg).*

Hordenine (N-N-dimethyltyramine) is a naturally occurring amine that is marketed as a stimulant and fat burner due to its similarities to other adrenergic amines such as tyramine. Hordenine is naturally found in bitter orange, barley, sprouted barley, and malt. Hordenine became popularized after it was detected in the serum and urine of racing horses that were fed sprouting barley. Studies have demonstrated that a measurable increase of racing performance is improbable, but this has not stopped the VMS industry from including the understudied compound from pre-workout formulas. The mechanism of hordenine metabolism is not fully understood. Research efforts have demonstrated that hordenine has a high affinity for monoamine oxidase-B (MAO-B) in the liver, where it is deaminated and affects the sympathetic nervous system (Barwell, Basma, Lafi, & Leake, 1989). Pharmacological models conducted on hordenine have demonstrated that hordenine acts indirectly as an adrenergic-like compound that is a noradrenaline reuptake inhibitor (Hapke & Strathmann, 1995). Studies conducted with animal models report that hordenine has a positive inotropic effect upon the heart, increases

blood pressure and peripheral blood flow volume, and inhibits gut movement (Hapke & Strathmann, 1995). Thus, the inclusion of 50mg hordenine in MH raises some red flags regarding the safety of the product. Currently, there are no scientific studies that pertain to hordenine's role as a safe and efficacious nutritional supplement.

*Picamilon (50mg).*

Picamilon (nicotinyll-gamma-aminobutyric acid) is a synthetic combination of niacin and gamma-aminobutyric acid that was formulated by the Soviet Union in 1969. In Russia, picamilon is a prescription drug with antianxiety and vasodilatory properties. Most studies exploring picamilon are conducted by Russian scientists. For example, Dorofeev and Kholodov (1991) reported that the synthetic compound can rapidly penetrate the blood-brain-barrier and is intensively absorbed by animal organs. Shockingly, picamilon was banned by the FDA on November 30, 2015. A statement released by the FDA claimed that:

“picamilon is a unique chemical entity synthesized from the dietary ingredients niacin and gamma-aminobutyric acid. Picamilon is absorbed into the body, crosses the blood-brain barrier and accumulates in the brain as a separate chemical entity...any products marketed as dietary supplement that declare picamilon as a dietary supplement are misbranded” (US Food and Drug Administration, 2015 p.1).

The MH formula that was used to conduct The Efficacy and Safety of Six-Weeks of Pre-Workout Supplementation in Resistance Trained Rats was purchased in May of 2015 and contained the banned substance. In response to this ban, an independent study was conducted in the United States that explored the quantification of picamilon in dietary supplements. The

researchers found that of the 31 picamilon supplements available for sale, 30 contained picamilon in quantities ranging from 2.7 to 721.5 mg per recommended daily serving (Avula et al., 2016). Considering the high degree of variability, the researchers concluded that consumers cannot obtain accurate information from supplement labels regarding the quantity of picamilon. The evidence confirming inaccurate ingredient labeling in nutritional supplements brings into question regarding the accuracy of the rest of the ingredients in MH.

*N-Methyl-L-tyramine (50mg).*

N-Methyl-L-Tyramine (NMT) is human trace amine and natural phenethylamine alkaloid that is marketed as a stimulant and focus enhancer. NMT is naturally found in barley, bitter orange, and beer; there is approximately 3mg of NMT per pint of beer. Researchers isolated NMT from beer and reported that NMT increased insulin and gastrin secretion (Yoko et al., 1999). In humans, NMT is produced by the N-methylation of tyramine via phenylethanolamine N-methyltransferase (Broadley, 2010). The ability of NMT to cross the blood-brain-barrier is attenuated by its low lipid solubility and short half-life. NMT is a weak alpha-2 adrenoceptor antagonist that stimulates increased concentration, heartrate, and vasoconstriction by preventing the breakdown of adrenergic, dopaminergic, and serotonergic neurotransmitters (Koda et al., 2001). NMT supplementation may result the reduction in noradrenaline responses because NMT is metabolized into octopamine—an inactive noradrenaline analogous that is packaged in place of noradrenaline in synaptic vesicles. The most commonly reported side effect of NMT supplementation is headaches (D'andrea, Nordera, Perini, Allais, & Granella, 2007).

According to the Banned Substances Control Group, NMT is prohibited in sport (Banned Substances Control Group, 2016). Thus, it raises concerns why NMT is included in MH when it

is marketed to athletes looking for a competitive edge. Despite the ban, the bodybuilding community has touted NMT as a replacement for 1,3-dimethylamylamine—the banned substance contained in the pre-workout supplement JACK3D that was responsible for Private Sparling’s death in 2013. The efficacy and safety of NMT in improving exercise performance is unknown because there have been no studies conducted in humans. Thus, there is not a recommended dosage and it is unknown how 50mg of NMT in MH truly behaves in the body. However, due to drug’s weak agonizing ability and inefficiency in crossing the BBB, there is most likely no significant advantage in supplementing NMT relative to the risks of consuming an unknown drug.

*Yohimbine Bark Extract (2mg).*

Yohimbine—a naturally occurring alpha-2 receptor antagonist—is the most well studied compound in Mr. Hyde’s ‘intensity matrix.’ Yohimbine promotes sympathetic activity and ameliorates noradrenaline release. The recommended dosage of yohimbine is .2mg/kg bodyweight; i.e. 14mg for a 150lb person. Moderate doses of yohimbine prior to a workout have been demonstrated to lower the respiratory quotient (the ratio of CO<sub>2</sub> produce to O<sub>2</sub> consumed) and boost lipolysis by blocking adipocyte alpha-2 adrenoceptors (McCarty, 2002). Both animal models and human trials have demonstrated yohimbine’s efficacy as a weight loss supplement. For example, one animal model demonstrated that yohimbine can suppress appetite in genetically obese and lean mice (Callahan, Beales, & Oltmans, 1984). In another study, Ostojic (2006) explored the effects on body composition and exercise performance in elite soccer players. Subjects (n=20) orally consumed 20 mg per day in two equal doses of placebo or yohimbine for 21 days. Data suggested no changes in exercise performance but demonstrated a

significant reduction in average body fat levels from 9.3% to 7.1% (Ostojic, 2006). Thus, yohimbine is an attractive compound to include in pre-workout supplements because it can stimulate weight loss.

The mechanism by which yohimbine stimulates weight loss—by increasing noradrenergic function—may also work to induce anxiety in individuals. Sommer et al., 2011 conducted a study that explored the psychological and neuroendocrine response to social stress and to the administration of yohimbine in highly trained endurance athletes (n=12) compared to untrained healthy controls (n=12). Both groups performed a challenge paradigm under the influence of .4mg/kg yohimbine, placebo, or a psychosocial stress test. The researchers quantified the responses to social stress through psychometric scales, plasma cortisol, blood pressure, and heart rate. The researchers found that yohimbine increased anxiety, cortisol, blood pressure, and heart rate in both cohorts. Specifically, cortisol only increased in the untrained persons whereas both groups experienced equal rates of anxiety (Sommer et al., 2011).

Kearney, Tu, and Haller (2010) conducted a retrospective review of the California Poison Control System reported cases to determine the prevalence and severity of adverse side effects caused by yohimbine. The researchers identified 238 cases of adverse side effects between 2000 and 2006. In 2000, the prevalence of yohimbine-related adverse drug events was 1.8 cases per 10,000 total adult exposures whereas the prevalence increased to 8 cases per 10,000 in 2006. The most common side effects were gastrointestinal distress, tachycardia, anxiety, and hypertension (Kearney et al., 2010). Interestingly, a genetic predisposition to yohimbine-related adverse side effects may exist. For example, individuals that are deficient in the *CYP2D6* gene—which codes for the enzyme that metabolizes yohimbine into 11-hydroxy-yohimbine—may experience

increased nervous system effects from yohimbine because it exists in their system longer (Le Corre et al., 2004).

The label on MH reports that each scoop contains 2mg of yohimbine; however, there is a chance that the actual content of yohimbine is mislabeled. Cohen, Wang, Maller, DeSouza, and Khan (2015) analyzed 49 brands that listed yohimbine on their ingredients list. The supplements were analyzed via liquid chromatography and mass spectrometry. The researchers reported that the quantity of yohimbine ranged from 0mg-12.1mg and that the actual content ranged from 23-147% of the content label (Cohen et al., 2015). As stated previously, this isn't the first case in which pre-workout formulas are inaccurately labeled. The irresponsible tendencies to mislabeled ingredients is especially dangerous, especially because yohimbine can cause adverse side effects to people with a genetic predisposition.

*Rauwolfia Vornitoria Root Extract (2mg).*

The active ingredient in *Rauwolfia vornitoria* root extract is rauwolscine. Rauwolscine is a diastereoisomer of yohimbine and functions similarly to yohimbine. For example, rauwolscine is an alpha-2-adrenergic receptor antagonist and fat burner. In rat models, rauwolscine has shown to have less affinity for alpha-2-adrenergic receptors and is consequently less potent than yohimbine in attenuating adrenaline-induced blood pressure increases (Rockhold and Gross, 1981). The different spatial configuration of rauwolscine makes it a 5-HT1a/b receptor agonist that can boost mood (Arthur, Casañas, & Raymond, 1993). There is not an abundance of literature relating rauwolscine and resistance training, and thus there is not a scientific consensus on the compounds efficacy and safety in resistance training.



*Why Mr. Hyde (MH)?*

The Efficacy and Safety of Six-Weeks of Pre-Workout Supplementation in Resistance Trained Rats specifically chose to explore the effects of MH on performance, muscle hypertrophy, vascular function, and renal function for a number of reasons. First, MH is one of the most popular PWSs on the market and it is logical to study a product that has a high prevalence of consumption. Consumers buy MH because it has a unique proprietary blend that is marketed to improve performance. Thus, a resistance training regimen was designed to explore the effects of MH supplementation on performance and muscle hypertrophy in rats. Next, the ‘caffeine matrix’ of MH has raised a red flag due to its unparalleled concentrations of caffeine (419mg). Such a high degree of caffeine imposes serious health risks (i.e. myocardial infarction) and may mitigate heart and vascular function. Another reason for selecting MH was due to the variety of that ingredients that have not undergone the scientific scrutiny. Many of the ingredients—especially those in MH’s ‘intensity matrix’—are understudied, lack proper consumption recommendations, contain banned compounds, and are inaccurately labeled. Furthermore, the efficacy, safety, and independent mechanisms of these ingredients are far from being elucidated. The human body is very dynamic and these supplements do not act in isolation. Thus, this study raises concern about the compounding effects of these ingredients and uses vascular reactivity tests and urinary creatinine tests as markers of biological safety.

## Methods

### *Animals.*

Male, 3 month-old, F344 rats were purchased and housed in an isolated room in the College of William and Mary campus animal facility. Animals were kept on a 12:12 hour light-dark cycle and fed ad libitum with Harlan 2018 Teklad Global 18% Protein Rodent Diet. Experiments were approved by the College of William and Mary's Animal Care and Use Committee and conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (National Institutes of Health, 1985). Rats were randomly divided into one of the following two treatment groups: a placebo control group (CTRL;  $n=12$ ) and a Mr. Hyde group (MH;  $n=12$ ). The CTRL group was orally dosed with a dextrose-water solution, while the MH group was orally dosed with a MH-water solution. Since 7.5g of Mr. Hyde is intended to be consumed by a 75.3kg human, dosages were readjusted each week to represent the correct proportions between the growing rats and the recommended serving size. The rats performed resistance training 30 minutes after they were orally dosed with the respective formula.

### *Training.*

A six-week resistance training regimen was designed where animals would exercise for two days, followed by one rest day—this pattern remained consistent throughout the six weeks. Resistance training consisted of climbing a 1 m wire ladder, at an 85° angle, with increasing weight added to the tail; a schematic of the ladder climbing is depicted in figure 2. Each training session featured three sets, with two repetitions, of ladder climbing with a one-minute rest period between each set. Resistance was initially established at 50% of body mass with 30 g increments added after each successful completing in the previous set. Weight was added to a tube and

attached to the rat's tail via Velcro. The rat began its next training session with the weight it successfully completed during its last training session.

Below is an example of a successful day of resistance training for rat 7, a MH rat. On day 2 of the resistance training regimen, Rat 7 weighed 193g. 30 minutes after being orally dosed a proportional amount of MH, Rat 7 was placed at the bottom of the ladder and had to climb up the ladder. A bucket of cold water was placed underneath the ladder and the rat's cage was placed on top of the ladder to encourage upward climbing. Before each rep, the rat was placed at the bottom of the ladder and climbed up with weight attached to its tail. Since rat 7 weighed 193g, its first set consisted of 50% of its body weight. Rat 7 successfully climbed the ladder twice and after one minute of rest, it was ready to move onto the next weight, 50% of body weight + 30 grams. Rat 7 successfully performed two reps and moved onto the next weight, 50% of body weight + 60 grams. Hence, rat 7 completed 3 sets of 2 reps and was ready to move up weight during its next training session.

Below is an example of an unsuccessful day of resistance training for rat 7, a MH rat. On day 7 of the resistance training regimen, Rat 7 weight 212g. 30 minutes after being orally dosed a proportional amount of MH, Rat 7 was placed at the bottom of the ladder with 50% + 120g attached to its tail. Rat 7 successfully completed the first set and moved onto the next set, consisting of 50% + 150g. Rat 7 successfully completed the second set and moved onto the third set. On the third set, Rat 7 had 50% + 180g attached to its tail. Rat 7 was successful on its first rep, but on the second rep it failed to climb the ladder. Thus, day 7 was marked as a failure for rat 7 and it had to use the same weight standards for day 8 of training

*Vascular Reactivity.*

The vascular reactivity protocol was modeled after a study conducted by Harris, Slack, Prestosa, and Hryvniak (2010)—Dr. Harris is the Principle Investigator of the current study:

“Twenty-four hours following the last exercise animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and femoral arteries [and aortas] were removed and placed in ice-cold saline solution. Vessels were cleaned of adherent fat and connective tissue and cut into two, 2 mm rings. The remainder of the femoral artery [and aortas] [were] frozen in liquid nitrogen and used for immunoblotting. The rings were mounted using small wires on stainless steel holders in muscle baths on a DMT610 myograph for isometric force recording. Muscle baths were filled with physiological salt solution (PSS) consisting of: 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH<sub>2</sub>PO<sub>4</sub>, 1.17 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.6 CaCl<sub>2</sub>, 14.9 mM NaHCO<sub>3</sub>, 5.5 mM dextrose, and 0.03 mM Na<sub>2</sub>EDTA heated to 37°C and aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The rings were stretched to an optimal resting tension (1.5 g) and allowed to stabilize for 1 h. After equilibration, constrictor responses to phenylephrine were determined using cumulative doses (10<sup>7</sup> to 10<sup>4</sup> M). Relaxation responses to cumulative doses of acetylcholine (ACh, 10<sup>7</sup> to 10<sup>4</sup> M) and sodium nitroprusside (SNP, 10<sup>9</sup> to 3 x 10<sup>4</sup> M) were determined following precontraction with 10<sup>4</sup> M phenylephrine with >30 min of recovery between each drug until the resting tension stabilized. The bathing medium was changed every 5 min during the recovery periods. Aortic dose response curves to phenylephrine (PE, 10<sup>7</sup>–10<sup>4</sup> M), sodium nitroprusside (SNP, 10<sup>8</sup>–10<sup>5</sup> M), and acetylcholine (ACh, 10<sup>7</sup>–3x10<sup>5</sup> M) were constructed” (Harris et al., 2010).

### *Renal Function.*

Twenty-four hours following the last exercise animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and urine samples were collected from the bladder. The samples were frozen in liquid nitrogen and used for a urinary creatinine test. A creatinine assay kit (MAK080 Sigma) was purchased from Sigma-Aldrich in August 2015. Creatinine concentration was determined by a coupled enzyme reaction, which results in a colorimetric (570 nm)/fluorometric ( $\lambda_{\text{ex}} = 535/\lambda_{\text{em}} = 587$  nm) product, proportional to the creatinine present.

### *Muscle Excision.*

Twenty-four hours following the last exercise animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The soleus, plantaris, flexor digitorum longus, and extensor digitorum longus were removed, weighed, and frozen in liquid nitrogen.

### *Statistical Analysis.*

The reported values represent means  $\pm$  standard error (SE). To determine whether there was a significant difference among groups after 6 weeks of resistance training, two-way ANOVA tests were used to analyze the results. A P-value of  $<0.05$  was used as a limit for statistical significance.

## **Results**

ANOVA revealed a significant ( $P < 0.05$ ) main effect of MH on amount lifted; in other words, cumulatively more weight was lifted each day in the MH groups than the control group, but no specific day was different and the maximal amount of weight at the end of six weeks was not significantly different (i.e. no post-hoc test for each individual time point revealed significance) (see figure 3). No significant differences ( $P > 0.05$ ) were observed in skeletal muscle weight (indicating no hypertrophy) (see figures 4-7). No significant differences ( $P > 0.05$ ) in

vascular function measured by dose response curves to PE, Ach, and SNP (no change in IC<sub>50</sub> or in the maximal response) (see figures 8-13). Urinary creatinine levels in the MH group were significantly ( $P<0.05$ ) increased (see figure 14). Taken together, these data suggest that 6 weeks of pre-workout supplementation in rats undergoing resistance training resulted in modest efficacy with improvement in weight lifted with no change in muscle size or vascular reactivity. The significant increase in urinary creatinine levels suggests potential renal health issues; however, more renal function tests need to be conducted for further elucidation of this claim.

## **Discussion**

A PubMed search for ‘pre-workout supplement’ and ‘resistance training’ yields ten items. An additional PubMed search for ‘pre-workout supplement’ and ‘safety’ yields eight items. In the following section, the relevant studies will be discussed as it pertains to the current study.

Martinez, Campbell, Franek, Buchanan, and Colquhoun (2016) explored the effect of acute pre-workout supplementation on power and strength performance. The researchers studied a popular supplement called MusclePharm Assault™. Figure 15 lists the supplement facts of the product. In a randomized, double-blinded, crossover design, subjects (n=13) reported to the laboratory on four separate occasions, each separated by one week. On the first occasion, subjects underwent a familiarization session where they were instructed how to complete a 24 h food log, standardized dynamic warm-up, and completion of the performance testing protocol that included a medicine ball put (MBP), vertical jump test (VJ), one-rep maximum bench press (1-RM), and a Wingate Anaerobic Power Test (WAnT). On the second occasion, subjects underwent a baseline trail where they measured their height, weight, and blood pressure, completed a 24-hour food log, and were guided through a standardized dynamic warm-up followed by completion of the performance testing protocol. On the third and fourth occasions,

subjects were randomly provided either the SUP or PL and performed the exercise protocol 20min after consuming the SUP or PL. The researchers noted that the SUP group yielded significant differences in WAnT, anaerobic mean power, and base line performance. However, no significant differences were observed for upper body power, upper body strength, or upper body strength (Martinez et al., 2016). Thus, the researchers concluded that acute ingestion of PWSs can significantly improve both anaerobic peak power and mean power in recreational trained males. The researchers also conceded to the fact that there is a scarcity of research on pre-workout supplements and that more research is warranted.

Kedia et al. (2014) explored the effects of a pre-workout supplement on lean mass, muscular performance, subjective workout experience, and biomarkers of safety. The researchers studied a popular pre-workout supplement known as Craze—in October 2013, Craze was banned after researchers discovered that N, $\alpha$ -diethyl-phenylethylamine (N, $\alpha$ -DEPEA), a methamphetamine analog was not listed on Craze's ingredient label. Figure 16 represents the ingredients label; notable ingredients include creatine, caffeine, betaine, and Dendrobex (an herbal medicine that might have blood pressure-lowering effects while serving as a stimulant, and a neurotropic (Kedia et al., 2014). The prospective, randomized, double-blind, trail consisted of two parts. In part 1, subjects (n=40) ingested one dose of SUP or PL and were measured for resting heart rate, blood pressure, ECG, and comprehensive blood chemistry and blood counts. The researchers noted a significant increase in systolic and diastolic blood pressures in the SUP group, whereas the PL group had non-significant reductions. In Part 2, subjects (n=43) underwent a six-week training regime with daily doses of SUP or PL. The researchers reported significant improvements in subjective energy and concentration. At week 6, body composition was measured via dual-energy x-ray absorptiometry (DEXA) and did not reveal improvements in

measures of body composition. Thus, the researchers concluded that pre-workout supplementation increased energy and concentration, but did not yield improvements in performance or body composition—similar to the Mr. Hyde study (Kedia et al., 2014).

Joy et al. (2015) examined the safety of one and two servings of the popular pre-workout supplement, MusclePharm Assault™. A unique part about the design of this study is that the researchers explored dose-escalation. This is a realistic approach because a lot of bodybuilders tend to consume more than one dose before their workout—despite the warning label—because they develop tolerances for the supplement. Interestingly, the exact formula was explored by Martinez et al. (2016) and the ingredients can be viewed in Figure 15. In a randomized design, subjects (n=44) were divided into an un-supplemented control group (CRL), one-serving group (G1), or two-serving group (G2). The subjects ingested the pre-workout supplement (or nothing at all) every day for 28 days in an un-blinded manner. Subjects were instructed to complete a log and maintain their habitual dietary and exercise routines throughout the 28-day study. A major flaw of this study is that the researchers did not make the subjects follow a set exercise routine or diet, and may account for a lot of variability. Prior to and following the supplementation period, the researchers measured vital signs and analyzed the samples for hematological and clinical chemistry panels. Vital sign analysis revealed significant group x time interactions for mean corpuscular hemoglobin, platelets, serum glucose, and albumin; however, the variables remained within the clinical reference ranges. Thus, the researchers concluded that both one-serving and two-servings consumed daily for 28 days was deemed to be safe for heart, liver, and kidney function (Joy et al., 2015).

Shelmadine et al. (2009) conducted a study that determined the effects of 28 days of heavy resistance exercise combined with the pre-workout supplement, NO-Shotgun®, on body



composition, muscle strength and mass, markers of satellite cell activation, and clinical safety markers. The researchers did not include a specific ingredients list in their paper, thus we cannot compare the ingredients to Mr. Hyde because the formulas constantly change and we cannot definitely confirm which formula of NO-Shotgun<sup>®</sup> was used. Despite this flaw in the study, the rest of the study displayed sound methodologies. In a double-blind, placebo controlled study, subjects (n=18) were placed on a 28day, 4times/week resistance training regimen that was split into two upper-extremity and two lower-extremity exercise sessions. The subjects were randomly divided into a NO-Shotgun group (NO) or a placebo group (PL) and were directed to ingest one scoop (27g) of NO-Shotgun<sup>®</sup> or PL 30 min prior to exercise. The researchers found that the NO group displayed a significant increase in fat-free mass, bench press strength, myofibrillar protein, serum hepatocyte growth factor, and total DNA content. The researchers also reported no significant differences in whole blood and serum clinical chemistry markers (Shelmadine et al., 2009). Thus, the researchers concluded that NO-Shotgun supplementation for 28 days in combination with heavy resistance training increases muscle strength and mass, myofibrillar protein content, and markers indicative of satellite cell activation without posing any negative side effects.

Outlaw et al. (2014) performed a double-blind, randomized, match-pair design study and evaluated the effects of a proprietary blend containing creatine monohydrate, beta-alanine, L- taurine, L-leucine, and caffeine on anaerobic power, muscular strength, body composition, and mood states. Subjects (n=20) were divided into two groups and consumed one scoop (30g) of the pre-workout supplements (which was not specifically named) or placebo 30 minutes before a resistance training workout. Subjects completed a baseline test followed by eight days of supplementation and performed a workout that consisted of 1RM and repetitions to failure of

bench press and leg press as well as 12 vertical jumps (VJ) and a Wingate power test on a cycle ergometer. The researchers found no significant improvements in body composition or performance; however, they did note trends indicative of improvement that is consistent with the findings in this study.

Jagim et al. (2016) conducted a double-blind, randomized, placebo controlled, crossover study that explored the effects of pre-workout supplements on strength performance and muscle hypertrophy. The study did not mention the name of the product, but included an ingredients list (see figure 17); notable ingredients in the product are creatine hydrochloride, caffeine anhydrous, and beta-alanine. The subjects (n=12) were consumed either SUP or PL 30 min prior to a workout that consisted of a counter movement vertical jump test, 5 sets of repetitions at 85% of 5RM of back squats and bench press, followed by a single set to failure, and an anaerobic capacity sprint test to assess peak and mean power. One week later, the subjects returned for a second testing session under the influence of the counter treatment (Jagim et al., 2016). Data revealed that the SUP group significantly increased in total volume load, mean power during the anaerobic spring test, and subjective markers of fatigue and alertness.

The relevant literature that explores the effects of pre-workout supplementation on exercise performance and safety establishes some consistent trends. First, all of the studies demonstrated that pre-workout supplementation enhances exercise performance—whether it is statistically significant or a trend. Second, the studies that explored biological markers of safety all conclude that there is not a clinically significant risk of acute pre-workout supplementation. While researching the acute effects is the first logical step in garnering a better understanding of pre-workout supplements, more studies need to be conducted in order to delineate the long term effects. The body of literature is scarce and we cannot infer a scientific consensus based off the

current data. There is a plethora of different pre-workout formulas sold throughout the United States and establishing the relative safety from a few products cannot be generalized to the entire industry. For example, the proprietary blend in Mr. Hyde contains many unique ingredients—especially in its ‘intensity matrix’—that have never been tested in human subjects. Thus, the current study is a novel study because it is the only study that independently analyzes Mr. Hyde. Furthermore, it is one of the only animal models in the scarce body of literature. While animal models cannot be directly applied to humans, using rats yields some advantages. For example, all of the rats were genetically similar and lived in a controlled environment. The reduction of environmental confounding factors allowed us to study Mr. Hyde in isolation. Another advantage of using an animal model was the opportunity to euthanize the rats, extract tissue, and run vascular reactivity tests, and follow up biochemical analysis of tissues. Currently, this study is the only study that analyzes vascular reactivity; although there was no significant difference, it still has pioneering characteristics.

## **Conclusion**

The Efficacy and Safety of Six-Weeks of Pre-Workout Supplementation in Resistance Trained Rats explores the effects of Pro Supps Mr. Hyde on muscle hypertrophy, renal function, and vascular reactivity. The Vitamins, Minerals, and Supplements (VMS) industry has is essentially unregulated by the FDA and has a history of mislabeling supplements, including banned substances, and causing adverse side effects to consumers. Pro Supps Mr. Hyde is just one example of a product that contained banned supplements—such as picamilon—and mislabeling ingredients—such as picamilon and yohimbine. Furthermore, the pre-workout supplement contains a proprietary blend of ingredients that have a scarcity of scientific evidence regarding their efficacy and safety. Some ingredients, especially those listed in the ‘intensity

matrix' of MH, have never been tested in human subjects. Thus, those who consume Mr. Hyde are analogous to lab rats because there is no way to determine how these ingredients independently combine in the body. What is even more troublesome is that there are virtually no studies that examine the biological synergisms of this unique proprietary blend. Hence, this study utilized an animal model to determine the efficacy and safety of Mr. Hyde. After 6-weeks of resistance training, there was no significant increase in exercise performance, or muscle hypertrophy—but there was a significant lift main effect that is congruent with other studies that study pre-workout supplements and resistance training. Regarding renal function, the Mr. Hyde group demonstrated an increase in urinary creatinine; however, after reviewing the literature surrounding creatine supplementation and creatinine levels, this trend is expected and is not a conclusive biological safety marker. Regarding vascular reactivity, there were no significant differences between the groups; however, the analysis of vascular reactivity and pre-workout supplementation is the first of its kind and serve a pioneering role. Hence, this study served a preliminary role to establish the efficacy and safety of six-weeks of preworkout supplementation in resistance trained rats. More studies need to be conducted to determine the long term effects of pre-workout supplementation and to analyze the interactions between the understudied ingredients in Mr. Hyde's proprietary blend.

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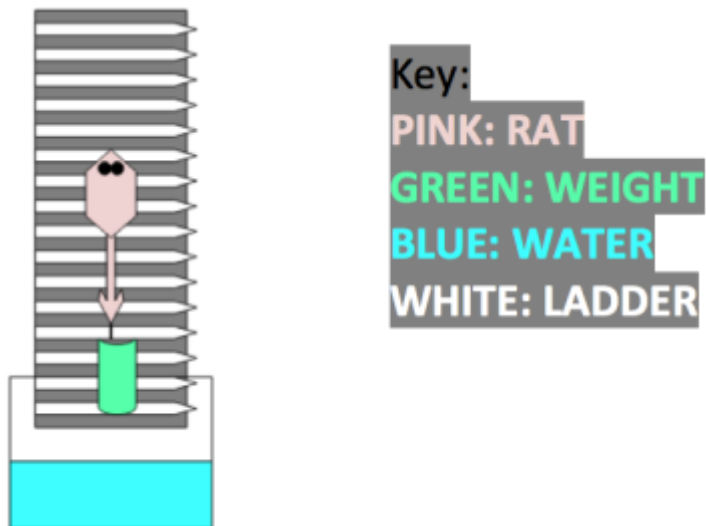
## Figures

<b>Supplement Facts</b>		
Serving Size: 1 Scoop (7.5g)		
Servings Per Container: 15		
	Amount Per Serving	% Daily Value
<b>Strength Matrix</b>	<b>5g</b>	<b>**</b>
Beta Alanine (CarnoSyn®)	2.5g	**
Creatine Nitrate (NO3-T®)	1g	**
L-Leucine	500mg	**
Agmatine Sulfate (AGMAPURE®)	500mg	**
L-Citrulline Aspartate (1:1)	500mg	**
<b>Caffeine Matrix</b>	<b>419mg</b>	<b>**</b>
Caffeine Anhydrous	300mg	**
Infinerty™ (Dicafeine Malate)	69mg	**
Caffeine Citrate	50mg	**
<b>Intensity Matrix</b>	<b>154mg</b>	<b>**</b>
Hordenine	50mg	**
Pikazopin® Picamilon	50mg	**
N-Methyl L-Tyramine HCl	50mg	**
Yohimbe Bark Extract	2mg	**
Rauwolfia Vomitoria Root Extract (std. min. 90% Alpha-Yohimbine)	2mg	**

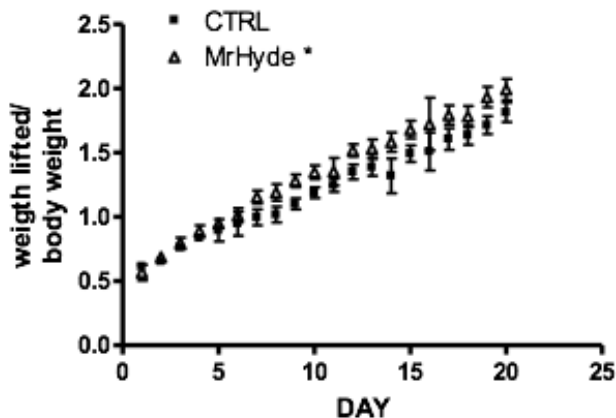
\*\* Daily Value not Established.

**Other Ingredients:** Natural & Artificial Flavor, Soluble Corn Fiber, Malic Acid, Sucralose, Calcium Silicate, Silicon Dioxide, FD&C Red #40, FD&C Red Lake #40, Soy Lecithin.      **Contains Soy Lecithin.**      **Contains 0% Juice.**

*Figure 1. Supplement Facts of Pro Supps Mr. Hyde purchased in May 2015:* MH is a unique proprietary blend that contains three matrices: a strength matrix, a caffeine matrix, and an intensity matrix. The strength matrix (5g) is composed of 2.5g beta alanine, 1g creatine nitrate, 500mg L-leucine, 500mg agmatine sulfate, and 500mg L-citrulline aspartate. The caffeine matrix (419mg) is composed of 300mg caffeine anhydrous, 69mg dicafeine malate, and 50mg caffeine citrate. The intensity matrix (154mg) is composed of 50mg hordenine, 50mg picamilon, 50mg N-methyl L-tyramine HCl, 2mg yohimbe bark extract, and 2mg rauwolfia vomitoria root extract.

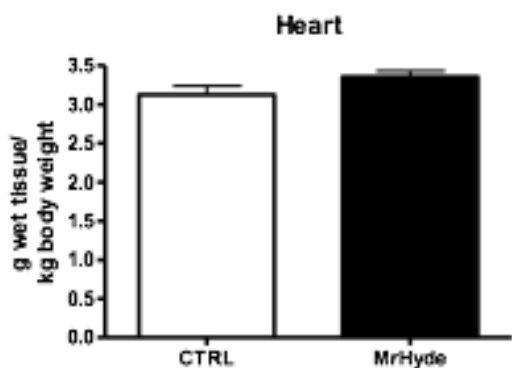


*Figure 2. Schematic of Training:* Animals climbed a 1 m ladder with 2-cm steps, inclined at  $85^\circ$  5 days/week for 6 weeks (2 work days followed by an off day). A cylinder containing weights was attached to the base of the tail and resistance was increased by adding weights to the cylinder.

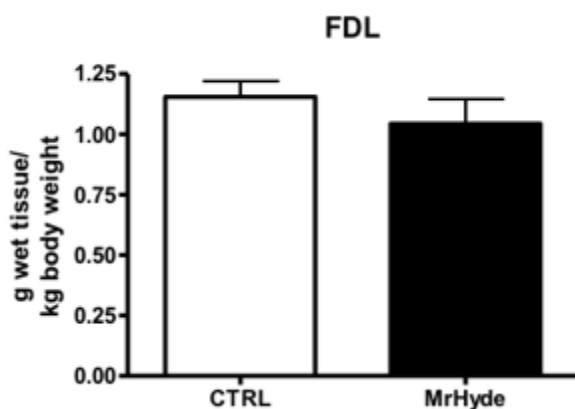


*Figure 3. Weight lifted/Body Weight vs Day of Training:* ANOVA revealed a significant ( $P < 0.05$ ) main effect of MH on amount lifted; in other words, cumulatively more weight was lifted each day in the MH groups than the control group but no specific day was different and the maximal amount of weight at the end of six weeks was not significantly different (i.e. no post-hoc test for each individual time point revealed significance).

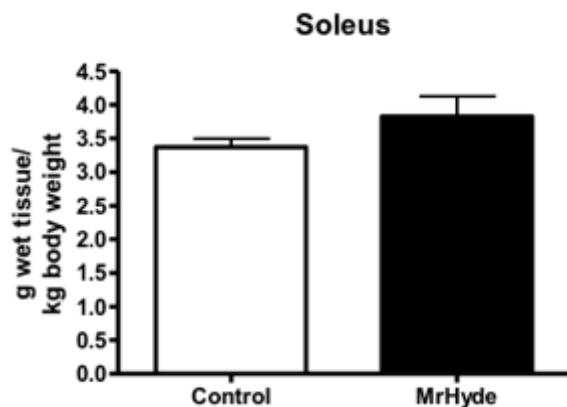




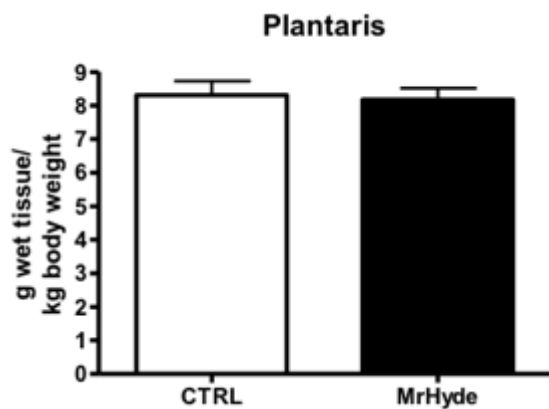
*Figure 4. Grams of Wet Heart Tissue / Kg Body Weight:* These data suggest that that six weeks of pre-workout supplementation in rats undergoing resistance training does not yield a significant difference ( $P>.05$ ) in heart mass.



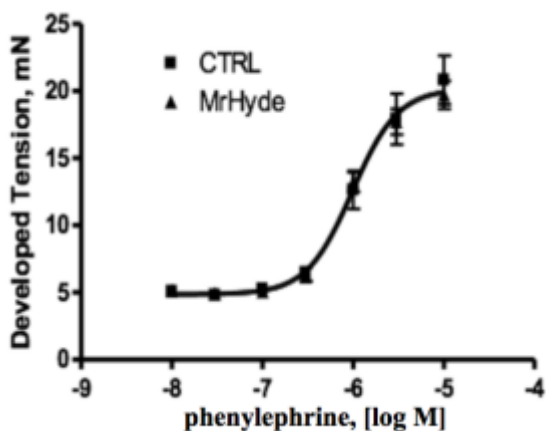
*Figure 5. Grams of Wet Flexor Digitorum Longus tissue / Kg Body Weight:* These data suggest that that six weeks of pre-workout supplementation in rats undergoing resistance training does not yield a significant difference ( $P>0.05$ ) flexor digitorum longus mass—indicating no hypertrophy.



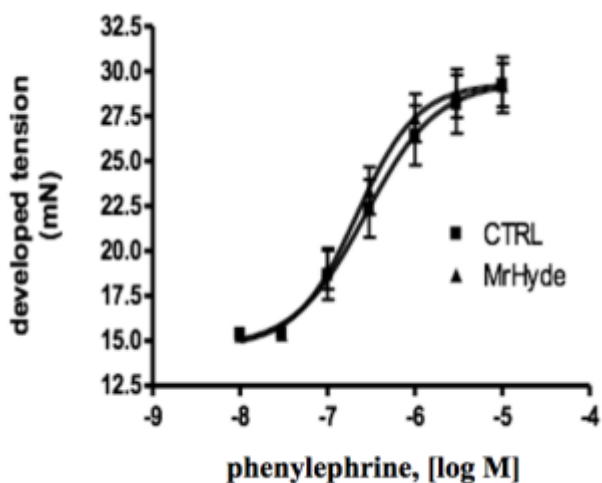
*Figure 6. Grams of Wet Soleus Tissue / Kg Body Weight:* These data suggest that that six weeks of pre-workout supplementation in rats undergoing resistance training does not yield a significant difference ( $P > 0.05$ ) in soleus mass—indicating no hypertrophy.



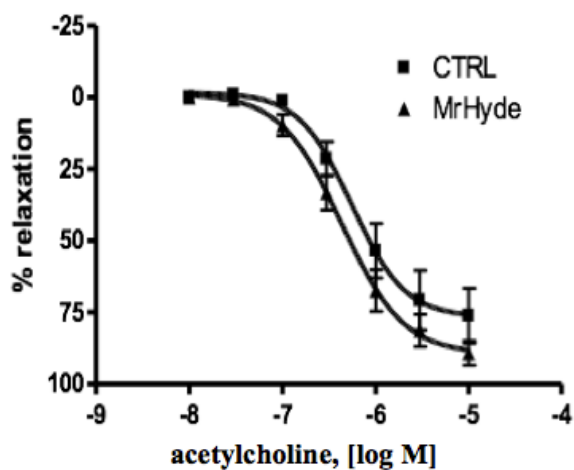
*Figure 7. Grams of Wet Plantaris Tissue / Kg Body Weight:* These data suggest that that six weeks of pre-workout supplementation in rats undergoing resistance training does not yield a significant difference ( $P > 0.05$ ) in plantaris mass—indicating no muscle hypertrophy



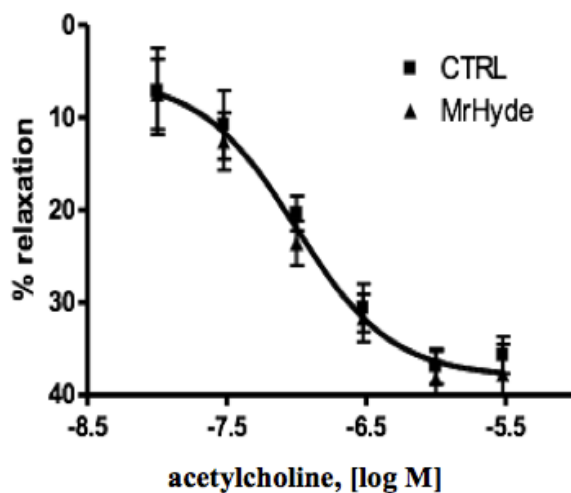
*Figure 8. Phenylephrine, femoral artery dose-response curve:* These data suggest there is no significant difference ( $P > 0.05$ ) in vascular function as measured by dose response curves to phenylephrine (no change in  $IC_{50}$  or in the maximal response) on the femoral arteries between CTRL and MrHyde.



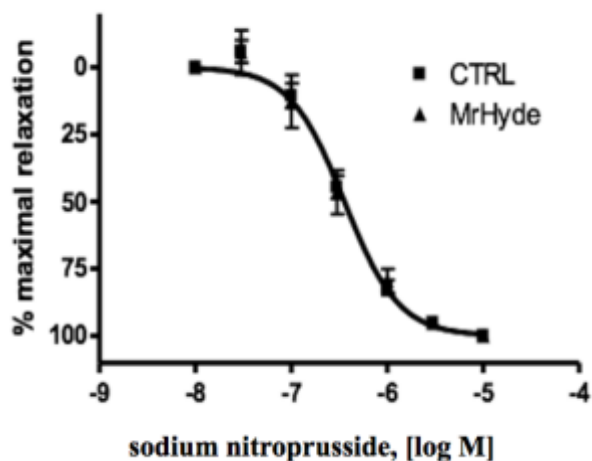
*Figure 9. Phenylephrine, aortic dose-response curve:* These data suggest there is no significant difference ( $P > 0.05$ ) in vascular function as measured by dose response curves to phenylephrine (no change in  $IC_{50}$  or in the maximal response) on the aorta between CTRL and MrHyde.



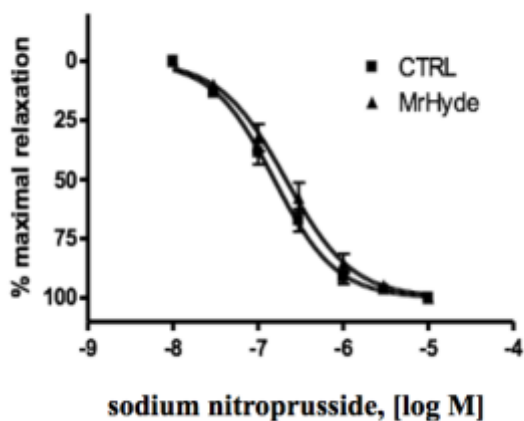
*Figure 10. Acetylcholine, femoral dose-response curve:* These data suggest no significant difference ( $P > 0.05$ ) in vascular function as measured by dose response curves to acetylcholine (no change in  $IC_{50}$  or in the maximal response) on the femoral arteries between CTRL and MrHyde.



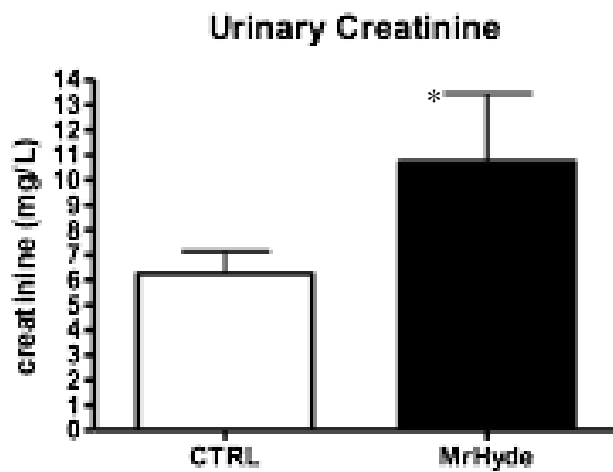
*Figure 11. Acetylcholine, aortic dose-response curve:* These data suggest no significant difference ( $P > 0.05$ ) in vascular function as measured by dose response curves to acetylcholine (no change in  $IC_{50}$  or in the maximal response) on the aorta between CTRL and MrHyde.



*Figure 12. Sodium Nitroprusside, aortic dose-response curve:* These data suggest no significant difference ( $P > 0.05$ ) in vascular function as measured by dose response curves to sodium nitroprusside (no change in  $IC_{50}$  or in the maximal response) on the femoral arteries between CTRL and MrHyde.



*Figure 13. Sodium Nitroprusside, femoral dose-response curve:* These data suggest no significant difference ( $P > 0.05$ ) in vascular function as measured by dose response curves to sodium nitroprusside (no change in  $IC_{50}$  or in the maximal response) on the aorta between CTRL and MrHyde.



*Figure 14. Creatinine (mg/L) levels between CTRL and MrHyde:* These data suggest that creatinine levels in the MrHyde group were statistically significant ( $p < .05$ ) compared to the CTRL group.

<b>Supplement Facts</b>		
Serving Size 14.5g (1 scoop)		
Servings Per Container: 30		
	Amount Per Serving	% DV†
<b>Calories</b>	<b>10</b>	
<b>Total Carbohydrates</b>	<b>3 g</b>	<b>1%</b>
<b>Sugars</b>	<b>2 g</b>	*
Vitamin C (Ascorbic Acid)	500 mg	833%
Vitamin E (as Alpha Tocopherol Acetate)	200 IU	667%
Niacin	60 mg	300%
Vitamin B6 (as Pyridoxine Hydrochloride)	15 mg	750%
Vitamin B12 (as Methylcobalamin)	90 mcg	1500%
Calcium (as Silicate, Phosphate and Citrate)	152 mg	15%
Sodium	50 mg	2%
Potassium	40 mg	1%
<b>ATP AMPLIFIER</b>	<b>3500 mg</b>	*
Carnosyn® Patented Beta-Alanine (2,000mg), L-Tyrosine, L-Aspartic Acid, Red Beet Extract High in Nitrates, (DMG) Dimethylglycine HCl.		
<b>CELLULAR TRANSPORT &amp; INSULIN ACTIVATOR</b>	<b>2952 mg</b>	*
Dextrose, L-Glycine, Ribose, Cinnulin PF® (Cinnamomum burmannii) Bark Extract.		
<b>ATHLETE PERFORMANCE BLEND</b>	<b>2000 mg</b>	*
<b>FEATURING "ION-3 NITRATE TECHNOLOGY MATRIX"</b> CreNitrate, ArgNitrate, BCAA Nitrate 3:1:2 Ratio (L-Leucine, L-Valine, L-Isoleucine).		
<b>ENERGY &amp; NEURO IGNITER</b>	<b>1750 mg</b>	*
Choline Bitartrate, Glucuronolactone, PurEnergy™ (caffeine), Huperzine A.		
<b>HYDRATION SYSTEM</b>	<b>1,200 mg</b>	*
Taurine, Coconut Water Powder, L-Glutamine Nitrate.		

†Percent Daily Values are based on a 2,000 calorie diet.  
\*Daily Value not established.

**Other Ingredients:** Natural & Artificial Flavors, Sucralose, Red Beet Juice Powder (for color), Citric Acid, Medium Chain Triglyceride Powder, Acesulfame Potassium, Beta Carotene (for color).

*Figure 15. Ingredients label of the popular PWS, MusclePharm Assault™:* Martinez, Campbell, Franek, Buchanan, & Colquhoun (2016) explored the effect of acute MusclePharm Assault supplementation on power and strength performance.

<b>45 Servings</b>		<b>Candy Grape</b>	
Serving Size: 1 Scoop (5.3 grams)			
Servings Per Container: 45			
<b>Amount Per Serving</b>		<b>% Daily Value</b>	
Vitamin C (Asorbic Acid)	250 mg	417%‡	
Kinesis™ Proprietary Blend	4,580 mg		*
Creatine Monohydrate, Trimethylglycine (Betaine Anhydrous), L-Citrulline, Dendrobex™ (Dendrobium Extract) (Stem) (Concentrated For Alkaloid Content Including Dendrobine, Dendroxine, Dendramine, B-Phenylethylamine, N,N-Dimethyl-B-Phenylethylamine, And N,N-Diethyl-B-Phenylethylamine), B-Phenylethylamine HCl, Citramine™ (Citrus Reticulata Extract) (Fruit) (Concentrated For N-Methyltyramine Content), Caffeine Anhydrous			
‡ Percent Daily Values are based on a 2,000 calorie diet.			
† Daily Value not established.			
<b>Other Ingredients:</b>			
Citric Acid, Malic Acid, Natural & Artificial Flavors, Sucralose, Acesulfame Potassium, FD&C Red #40, FD&C Blue #1			
Allergen Information: This product was manufactured on equipment that processes products that may contain milk, egg, soy, tree nuts, peanuts, wheat, fish, crustaceans & shellfish.			

*Figure 16. Ingredients label of the popular PWS, Craze™: Kedia et al. (2014) explored the effects of a Craze supplement on lean mass, muscular performance, subjective workout experience, and biomarkers of safety.*



Serving Size: 1 Scoop (26.5 g)	
Amount Per Serving	Amount
Calories	15
Total Carbohydrates	4 g
Sugars	2 g
Sodium (as sodium chloride)	130 mg
Creatine Hydrochloride	2 g
CarnoSyn® Beta-Alanine	2 g
Betaine (Trimethylglycine)	1.5 g
Taurine	1 g
N-Acetyl L-Cysteine	600 mg
AlphaSize® Alpha-Glycerol Phosphoryl Choline	300 mg
Citrulline Malate	6 g
Beta vulgaris L. (beet root extract)	500 mg
L-Leucine	3 g
L-Isoleucine	1.5 g
L-Valine	1.5 g
L-Tyrosine	1.5 g
Caffeine Anhydrous	300 mg
Huperzine A	50 mcg
Bioperine® (Piper Nigrum Fruit Extract)	5 mg

*Figure 17. Ingredients label of the unnamed PWS: Jagim et al. (2016) to explore the effects of pre-workout supplements on strength performance and muscle hypertrophy.*

## Appendices

### Appendix A. Resistance training schedule for all 24 rats:

JUNE 2015

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
31	1	2	3	4	5	6
7	8	9	10	11	12	13
14	15	16	17	18	19	20
	1,2	1,2,3,4	3,4,5,6	1,2,5,6,7,8	1,2,3,4,7,8,9,10	3,4,5,6,9,10,11,12
21	22	23	24	25	26	27
1,2,5,6,7,8,11,12, 13, 14	1,2,3,4,7,8,9,10,13,14,15,16	3,4,5,6,9,10,11,12,15,16,17,18	1,2,5,6,7,8,11,12,13,14,17,18,19,20	1,2,3,4,7,8,9,10,13,14,15,16,19,20,21,22	3,4,5,6,9,10,11,12,15,16,17,18,21,22,23,24	1,2,5,6,7,8,11,12,13,14,17,18,19,20,23,24
28	29	30	1	2	3	4
1,2,3,4,7,8,9,10,13,14,15,16,19,20,21,22	3,4,5,6,9,10,11,12,15,16,17,18, 21, 22, 23, 24	1,2,5,6,7,8,11,12,13,14,17,18,19,20,23,24	1,2,3,4,7,8,9,10,13,14,15,16,19,20,21,22	3,4,5,6,9,10,11,12,15,16,17,18,21,22,23,24	1,2,5,6,7,8,11,12,13,14,17,18,19,20,23,24	1,2,3,4,7,8,9,10,13,14,15,16,19,20,21,22

## JULY 2015

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
<b>28</b>	<b>29</b>	<b>30</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
1,2,3,4,7,8,9,10,13,14,15,16,19,20,21,22	3,4,5,6,9,10,11,12,15,16,17,18,21,22,23,24	1,2,5,6,7,8,11,12,13,14,17,18,19,20,23,24	1,2,3,4,7,8,9,10,13,14,15,16,19,20,21,22	3,4,5,6,9,10,11,12,15,16,17,18,21,22,23,24	1,2,5,6,7,8,11,12,13,14,17,18,19,20,23,24	1,2,3,4,7,8,9,11,13,14,15,16,19,20,21,22
<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>
3,4,5,6,9,10,11,12,15,16,17,18,21,22,23,24	1,2,5,6,7,8,11,12,13,14,17,18,19,20,23,24	1,2,3,4,7,8,9,10,13,14,15,16,19,20,21,22	3,4,5,6,9,10,11,12,15,16,17,18,21,22,23,24	1,2,5,6,7,8,11,12,13,14,17,18,19,20,23,24	1,2,3,4,7,8,9,10,13,14,15,16,19,20,21,22	3,4,5,6,9,10,11,12,15,16,17,18,21,22,23,24
<b>12</b>	<b>13</b>	<b>14</b>	<b>15</b>	<b>16</b>	<b>17</b>	<b>18</b>
1,2,5,6,7,8,11,12,13,14,17,18,19,20,23,24	1,2,3,4,7,8,9,10,13,14,15,16,19,20,21,22	3,4,5,6,9,10,11,12,15,16,17,18,21,22,23,24	5,6,7,8,11,12,13,14,17,18,19,20,23,24	7,8,9,10,13,14,15,16,19,20,21,22	9,10,11,12,15,16,17,18,21,22,23,24	11,12,13,14,17,18,19,20,23,24
<b>19</b>	<b>20</b>	<b>21</b>	<b>22</b>	<b>23</b>	<b>24</b>	<b>25</b>
13,14,15,16,19,20,21,22	15,16,17,18,21,22,23,24	17,18,19,20,23,24	19,20,21,22	21,22,23,24	23,24	
<b>26</b>	<b>27</b>	<b>28</b>	<b>29</b>	<b>30</b>	<b>31</b>	<b>1</b>

*Appendix B. Vascular Ring Experiments for MrHyde:*

1. Turn on myograph heat
2. Place 1x KPSS and 1x PSS in water baths
3. Bubble PSS with O<sub>2</sub>
4. Rinse wells with dH<sub>2</sub>O
5. Add 6.5 ml PSS to each well
6. Mount 2 femoral rings and 2 aorta rings
7. Allow to stabilize at 5 mN(F) and 20 mN(A)
8. Add 6.5 ml KPSS + 6.5  $\mu$ l 10<sup>-2</sup> PE(F) and 6.5 ml KPSS + 6.5  $\mu$ l 10<sup>-3</sup> PE(A)  
**Wait 3 minutes**
9. Wash with PSS 4Xs  
**Wait 5 minutes**
10. Add 6.5 ml PSS + 6.5  $\mu$ l 10<sup>-2</sup> PE(F) and 6.5 ml PSS + 6.5  $\mu$ l 10<sup>-3</sup> PE(A)  
**Wait 3 minutes**
11. Wash with PSS 4Xs  
**Wait 5 minutes**
12. Add 6.5 ml KPSS  
**Wait 3 minutes**
13. Wash with PSS 4Xs  
**Wait 5 minutes**
14. Add 6.5 ml KPSS  
**Wait 3 minutes**
15. Wash with PSS 4Xs  
**Wait 5 minutes**
16. Wait until returns to resting tension or below (if below readjust)
17. Phenylephrine (PE) dose response curve  
**(wait approximately 3 min between doses or until force levels off)**

FEMORAL ARTERIES			AORTAS		
[Final] M	[Stock] M	$\mu$ l Added to 6.5 ml	[Final] M	[Stock] M	$\mu$ l Added to 6.5 ml
10 <sup>-7</sup>	10 <sup>-4</sup>	6.5	10 <sup>-8</sup>	10 <sup>-5</sup>	6.5
3x10 <sup>-7</sup>	10 <sup>-3</sup>	1.3	3x10 <sup>-8</sup>	10 <sup>-4</sup>	1.3
10 <sup>-6</sup>	10 <sup>-3</sup>	4.55	10 <sup>-7</sup>	10 <sup>-4</sup>	4.55
3x10 <sup>-6</sup>	10 <sup>-2</sup>	1.3	3x10 <sup>-7</sup>	10 <sup>-3</sup>	1.3
10 <sup>-5</sup>	10 <sup>-2</sup>	4.55	10 <sup>-6</sup>	10 <sup>-3</sup>	4.55
3x10 <sup>-5</sup>	10 <sup>-1</sup>	1.3	3x10 <sup>-6</sup>	10 <sup>-2</sup>	1.3
10 <sup>-4</sup>	10 <sup>-1</sup>	4.55	10 <sup>-5</sup>	10 <sup>-2</sup>	4.55

18. Washout 3-4 times with PSS, allow to return to baseline

19. Pre-constrict with  $6.5 \mu\text{l } 10^{-2}$  PE(F) and  $6.5 \mu\text{l } 10^{-3}$  PE(A)

**Wait until level**

20. Acetylcholine (Ach) dose response curve

**(wait approximately 3 min between doses or until force levels off.**

**(NOTE: femorals will relax and begin to constrict again)**

FEMORAL ARTERIES			AORTAS		
[Final] M	[Stock] M	MI Added to 6.5 ml	[Final] M	[Stock] M	$\mu\text{l}$ Added to 6.5 ml
$10^{-7}$	$10^{-4}$	6.5	$10^{-9}$	$10^{-6}$	6.5
$3 \times 10^{-7}$	$10^{-3}$	1.3	$3 \times 10^{-9}$	$10^{-5}$	1.3
$10^{-6}$	$10^{-3}$	4.55	$10^{-8}$	$10^{-5}$	4.55
$3 \times 10^{-6}$	$10^{-2}$	1.3	$3 \times 10^{-8}$	$10^{-4}$	1.3
$10^{-5}$	$10^{-2}$	4.55	$10^{-7}$	$10^{-4}$	4.55
$3 \times 10^{-5}$	$10^{-1}$	1.3	$3 \times 10^{-7}$	$10^{-3}$	1.3
$10^{-4}$	$10^{-1}$	4.55	$10^{-5}$	$10^{-2}$	4.55

21. Washout 3-4 times with PSS, allow to return to baseline

22. Pre-constrict with  $10^{-5}$  PE(F) and  $10^{-6}$  PE(A)

**Wait until level**

23. Sodium Nitroprusside(SNP) dose response curve

**(wait approximately 3 min between doses or until force levels off.**

**(NOTE: femorals will relax and begin to constrict again)**

FEMORAL ARTERIES			AORTAS		
[Final] M	[Stock] M	$\mu\text{l}$ Added to 6.5 ml	[Final] M	[Stock] M	$\mu\text{l}$ Added to 6.5 ml
$10^{-8}$	$10^{-5}$	6.5	$10^{-9}$	$10^{-6}$	6.5
$3 \times 10^{-8}$	$10^{-4}$	1.3	$3 \times 10^{-9}$	$10^{-5}$	1.3
$10^{-7}$	$10^{-4}$	4.55	$10^{-8}$	$10^{-5}$	4.55
$3 \times 10^{-7}$	$10^{-3}$	1.3	$3 \times 10^{-8}$	$10^{-4}$	1.3
$10^{-6}$	$10^{-3}$	4.55	$10^{-7}$	$10^{-4}$	4.55
$3 \times 10^{-6}$	$10^{-2}$	1.3	$3 \times 10^{-7}$	$10^{-3}$	1.3
$10^{-5}$	$10^{-2}$	4.55	$10^{-6}$	$10^{-3}$	4.55

24. END and CLEAN-UP (**SAVE FILE!**)

25. Remove tissues from myograph discard in trash

26. Rinse wells with water

27. Rinse with 8% acetic acid (wipe wells with swab)

28. Rinse 2 x with water (dry outside of wells with Kimwipe)

**29. TURN OFF OXYGEN TANK!! (and myograph)**