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Hunger Games: The Effects of Alternate Day Fasting on Food Intake, Body Weight, and Leptin and Ghrelin in Rats

Debra Kirsty Mortel Tacad
University of Nevada, Las Vegas

HUNGER GAMES: THE EFFECTS OF ALTERNATE DAY FASTING ON FOOD INTAKE, BODY WEIGHT, AND LEPTIN AND GHRELIN IN RATS

By

Debra Kirsty Mortel Tacad

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Debra Kirsty Mortel Tacad

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Department of Kinesiology and Nutrition Sciences

John Young, Ph.D.
Examination Committee Chair

Kathryn Hausbeck Korgan, Ph.D.
Graduate College Interim Dean

Laura Kruskall, Ph.D.
Examination Committee Member

James Navalta, Ph.D.
Examination Committee Member

Richard Tandy, Ph.D.
Examination Committee Member

Robbin Hickman, PT
Graduate College Faculty Representative

ABSTRACT

PURPOSE: To determine whether a compensatory increase in food intake occurs following a day of fasting, and to determine whether leptin and/or ghrelin levels change in response to ADF compared with *ad libitum* feeding.

INTRODUCTION: Recently, alternate-day fasting (ADF) has grown in popularity as an alternative to continuous energy restriction (CER) diets as a method for improving health and controlling food intake. Leptin and ghrelin are two hormones implicated in the regulation of food intake and body weight, however their response to ADF is unclear.

METHODS: Male Wistar rats of the same age and weight were randomly assigned to the ADF group (n=7) or a control group (n=7). The ADF group had alternating 24-hour fasting and feeding days for 30 days. Fasting rats were limited to 3-5g of regular chow on fasting days and were given food ad libitum on feeding days. The control group had food ad libitum everyday for the same 30 days. Food intake for each animal was measured on a digital scale and recorded daily, and body weight was measured weekly. 600 µl blood was taken from the tail of each rat at the end of day 1, day 2, day 29, and day 30, to measure hormone levels after a fasting and feeding day before and after the ADF intervention trial. Leptin and ghrelin levels were determined by radioimmunoassay.

RESULTS: Food intake by ADF rats was increased by 20% on ad libitum feeding days ($F = 3.484$, $p = .047$), however ADF rats had lower rates of weight gain compared to the control group (290 g vs 355 g; $F = 41.604$, $p < .001$). Total percent body fat was significantly higher in the ADF group compared with the control group (19.9% vs 15.6%; $t = -2.848$, $p = .015$). Diet had no significant effect on leptin levels ($F = .134$, $p = .895$). Ghrelin concentration was significantly lower on the last feeding day and significantly higher on the subsequent fasting day

in the ADF animals at the end of the study compared with starting values (854.8 → 1475.3 pg/ml (post) vs 1085.9 → 1306.6 pg/ml (pre); t = -3.97; p = .007).

CONCLUSION: Results of this study indicate that food intake on feeding days did not sufficiently increase to offset the calorie deficit incurred on fasting days. Despite a decrease in weight gain, percent body fat increased in the ADF group. This appears to be the result of an adaptive change in ghrelin sensitivity in response to the alternate day fasting regimen.

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CHAPTER 1

INTRODUCTION

Weight loss continues to be a growing concern for many people in industrialized countries. Key components in weight loss are increases in daily physical activity level and diet modifications including appetite control. Though consumers have become more concerned with "healthy" and "fresh" foods, most highly available and affordable foods tend to be those that are high in calories. Easy access to high calorie foods, combined with multi-media advertisements make appetite control one of the harder obstacles to overcome when trying to lose weight. Though appetite and hunger are sometimes used interchangeably, there are distinct differences between the two. Hunger is defined as the physiological need for food due to depletion of nutrients and is associated with discomfort or weakness caused by lack of food. Appetite is the desire to eat food, which can be separate from hunger, and is influenced by the external factors such as the smell of food being cooked, food advertisements, social settings, etc. Controlling food intake, regardless if it is induced by hunger or appetite, is the key to successfully sustaining weight loss.

Recently, intermittent fasting (IF) has grown in popularity as an alternative to continuous energy restriction (CER) diets as a method for improving health and controlling food intake. CER requires individuals to cut caloric intake by 20-40% every day while maintaining daily meal frequency [1]. Many deviations of IF exist, however alternate-day fasting (ADF) has become a popular variation in the past decade. In ADF, food is completely removed or extremely reduced (less than 500 kcals/day) for 24 hours on one day, followed by a non-fasting day where food is consumed *ad libitum*. The ability to eat freely on feeding days without having to count calories becomes a very attractive idea for most people. The main assumption of this diet is that

most people cannot consume twice their daily caloric need on the feeding days, thus lowering caloric intake over time. Though fasting for 24 hours seems difficult, advocates of the diet say that their desire to eat lessens on fasting days, and studies have shown high compliance during short-term ADF trials [2]. Both ADF and CER have been shown to promote weight loss, extend lifespan, and protect against insulin disorders, cardiovascular disease, and neurological disorders in mice [3]. However, studies on the effects of ADF on hormones related to food intake have shown mixed results [2, 4-16]. A few studies[2, 4-6, 11-14, 17, 18] have examined the effects of ADF on leptin and ghrelin in relation to chronic diseases such as diabetes and cardiovascular disease, but no studies have examined the possible interactions of these hormones and their relation to food intake.

While food intake can be affected by a number of factors, certain circulating hormones can influence a person's energy expenditure and desire to eat. Hormones secreted from the stomach (cholecystokinin, ghrelin, peptide YY), the pancreas (glucagon, insulin), and adipose tissue (adiponectin, IL-6, leptin, resistin) all play a role in appetite regulation and nutrient metabolism [19]. The "hunger hormone" ghrelin has been shown to promote fat deposition, and stimulate AMP kinase and neuropeptide Y (NPY) expression, thus eliciting food intake. Conversely, the "satiety hormone" leptin has been shown to increase fat metabolism and inhibit AMP kinase and NPY expression, which suppresses feeding [20, 21]. This study will focus on leptin and ghrelin because of the strong influence these hormones have on regulating food intake and body weight [22].

The purpose of this study is to determine whether a compensatory increase in food intake occurs following a day of fasting. A second purpose is to determine whether leptin and/or ghrelin levels change in response to ADF compared with *ad libitum* feeding.

Though several ADF studies have been done with human subjects, this study will focus on the hormonal response in rats. Using a rodent model for this experiment will allow for control of physical activity, calorie intake and nutrient intake so that just the effects of the fasting and refeeding trial can be observed.

Research Question 1: Do rats increase food intake on fed days to offset energy deficits incurred on fasted days?

H_0: Rats will increase food intake on fed days to offset energy deficits on fasted days.

H_1: Rats will not increase food intake on fed days to offset energy deficits on fasted days.

Research Question 2: Do leptin levels change in response to ADF compared with ad libitum feeding?

H_0: There will be no significant difference in plasma leptin levels between the ad libitum fed group and ADF group.

H_1: There will be significantly different plasma leptin levels in the ADF group compared with the ad libitum fed group.

Research Question 3: Do ghrelin levels change in response to ADF compared with ad libitum feeding?

H_0: There will be no significant difference in plasma ghrelin levels between the ad libitum fed group and ADF group.

H_1: There will be significantly different plasma ghrelin levels in the ADF group compared with the ad libitum fed group.

CHAPTER 2

LITERATURE REVIEW

Fasting and Alternate-day fasting

Fasting has been a common practice for many through integration in religious practices (ex. Ramadan, Lent, Yom Kippur). By definition, fasting is considered low or no calorie intake for a time period of 12 hours to a few weeks. Some physicians prescribe very low calorie fasting periods lasting over a week, but patients need to be closely monitored in specialized clinics [1]. Few "periodic fasting" diets require subjects to fast for two or more consecutive days with the fasts at least 1 week apart to keep weight from decreasing too dramatically. However, it is thought that intermittent fasts (IF) can induce similar benefits to those found in the longer periodic fasting diets due to the high frequency of the fasting cycles [1, 23].

Many variations of IF exist which are sometimes coupled with very constrained eating habits. Some variations involve fasting for part of the day, about 12-20 hours, followed by a highly regimented food intake for the remaining hours (ex. *Leangains*, *The Warrior Diet*). Other IF options have longer, less frequent fasting periods (only 1-2 times per week), which then are followed by an unrestricted eating for the rest of the week (*Eat Stop Eat* diet). The effect of differing variations of IF on lifespan in rats was tested [24]. Wistar rats (N=137) aged 42 days were distributed between 4 groups based on littermates and gender: control, fasting 1 in 4 days, fasting 1 in 3 days, and fasting 1 in 2 days. Rats were continued on their assigned diet until death. Results showed that the extension of life by fasting was almost proportional to the amount of fasting, however more complications were found in 1 in 4 days and 1 in 2 days IF groups. The lifespan of male rats improved more than females (90 days and 23 days, respectively), but female rats lived longer on average. Interestingly, the weights of IF rats were lower than the control

group with no significant retardation of growth in the rats. This was one of the first studies to compare different IF diets in relation to prolonged lifespans. The authors also noted that the IF treatments may help delayed the development of mammary tumors and other types of tumors compared to littermates in the control group, which could lead to longer lifespans as well. The ADF diet, also known as "alternate day calorie restriction" or "every other day diet," has been gaining significant scientific interest due to its comparable health benefits as continuous energy restriction (CER), but with higher dietary adherence in patients [2, 25, 26]. Though ADF was not tested in this experiment, its benefits on aging have been reviewed in both rodents and humans.

The exact causes of aging are currently unknown, however the prevailing theory is that oxidative stress causes damage within the cells leading to dysfunction and deterioration of cell [27]. When energy is produced during normal aerobic metabolism, reactive oxygen species (ROS) are formed as a byproduct. ROS are unstable oxygen radicals that have a higher reactivity than regular oxygen molecules. Though ROS play a role as signaling molecules for specific reactions, elevated levels can cause damage to lipids, proteins, and DNA. During aging, antioxidant and repair mechanisms that are needed to remove the ROS gradually become impaired. In mice, ADF has shown to help prevent age-related damage to antioxidant enzymes in bodily tissues. Coenzyme Q is an essential enzyme found in cell membranes and plays an important role in cellular metabolism and antioxidant activities [28]. Coenzyme Q may modulate a signaling pathway in the plasma membrane by reducing lipid peroxidation and certain coenzyme Q-dependent pathways can be effect by diet and exercise. The differing effects of age and diet were observes in male Swiss-OF1 mice aged 1-month and 11-month. Mice were then randomly assigned to four experimental groups: ad libitum sedentary (AL), ad libitum active

(ALT), ADF sedentary (EOD), and ADF active (EODT). After 6 months, no significant differences in coenzyme Q concentrations were found between the young and old groups. However, older mice in the EOD and EODT groups had significantly higher total coenzyme Q concentrations than the old AL group and young mice in the same group. The EOD and EODT groups also showed significant decreases in lipid peroxidation. Decreased rates of lipid peroxidation were also found in a similar experiment, which focused on the effects of ADF on antioxidant activity in 3.5-month-old female Wistar rats [29]. Combined these studies show a potential role for ADF in antioxidant protection.

Furthermore, ADF in rodent models has been shown to protect neurons against oxidative stress in neurodegenerative disorders, such as Alzheimer's and Parkinson's disease [30]. Positive effects of ADF are not just protective; ADF can also affect chronic issues. Johnson et al. studied the effects of ADF in obese asthmatic patients on symptoms, pulmonary function, oxidative stress, and inflammation [26]. Significant improvements in asthma-related symptoms and pulmonary functions were seen with the first 2 weeks and continued to improve until the end of the study. Participants had significant decreases in inflammatory and oxidative stress markers, as well as serum cholesterol and triglycerides.

The possible benefits of intermittent fasting/energy restriction and ADF on cardiovascular health and glucose metabolism have been extensively reviewed [12, 25] . To date, both human and rodent studies have shown IF and ADF can promote weight loss and improve health factors such as: insulin/insulin resistance, fasting blood glucose, and blood lipids profiles. In mice, modified ADF has been shown to modify body fat distribution, triglyceride metabolism, and adipokine secretion in a way that could be cardioprotective regardless of fat intake [13]. Male mice (N=18) were randomly assigned to one of three groups for four weeks: alternate day

fasting with low fat diet (ADF-LF), alternate day fasting with high fat diet (ADF-HF), and control group. Unlike many ADF studies in rodents, food restriction on fasting days was only cut by 85% (instead of 100%) and then fed ad libitum on feeding days. Though there were no significant differences in body weight between all three groups, both ADF groups had significantly greater amounts of subcutaneous fat and significantly lower percentages of visceral fat compared to control groups. Interestingly, mean energy intake between ADF-LF and ADF-HF was similar, despite differences in macronutrient composition. The adipokines leptin and resistin showed significant reductions in plasma concentrations of both ADF groups when compared to control group, though the ADF-LF group had greater reductions than the ADF-HF group. The combination of decreased visceral fat and the pro-inflammatory facilitator resistin promote ADF as method to protect against cardiovascular disease and atherosclerosis, even in the presence of a high fat intake in rodents. Similar improvements in adipose tissue physiology after a period of modified ADF has been seen in a human trial looking at the same parameters [18].

Harvie et al. compared the responses in whole body glucose metabolism and lipid profile after six months of ADF or CER in overweight women [8]. Subjects had comparable decreases in total cholesterol, LDL cholesterol, and triglycerides, but the ADF group had a greater decrease in fasting insulin levels, meaning this group had greater improvements in glucose metabolism than CER group. Prior to this, a similar study was done in eight non-obese males on an IF regimen of fasting for 20 hours every two days [31]. After 14 days, subjects maintained a constant weight and saw an increase in insulin-mediated glucose uptake and inhibition of adipose tissue lipolysis. However, no significant changes in fasting insulin levels were detected, which could be due to the short study length.

Negative effects of ADF have only been shown in two rodent studies. After eight months of ADF obesity-prone rats had an increase in reactive oxygen species (ROS), which increases inflammation and could lead to chronic disease development [32]. The other adverse reaction to ADF was seen in rats on a six month ADF trial, in showed significant decreases in diastolic compliance and increased myocardial fibrosis were found [4].

As seen above, the effects of ADF on health is still unclear. Some studies have demonstrated positive effects [12, 25, 26, 28-30], while others have shown no effect on health markers [4, 8, 9, 31, 32]. Understanding the mechanisms responsible for these effects are necessary for determining the efficacy and safety of this diet. In a review, Varady and Hellerstein note that weight seems to be the main confounding variable in ADF studies in both rodent and human models [12]. It is known that weight loss in obese populations can improve health and decrease risk for disease, however not all ADF studies showed decreases in weight. Studying the influence of ADF on appetite and appetite-related hormones could provide an answer to those conflicting results.

AMP Kinase Regulates Food Intake

AMP kinase is an enzyme that is expressed in many tissues, including brain, liver, cardiac and skeletal muscles. AMP kinase activation in the body can be affected by physiological and pathological stresses that increase ATP consumption or inhibit ATP production [19, 33]. AMP kinase acts as a sensor of cellular energy status by measuring the adenosine monophosphate (AMP) to adenosine triphosphate (ATP) ratio. On the cellular level, ATP is converted to ADP (adenosine diphosphate) when energy is needed. Two ADP molecules are then

transformed into one ATP and one AMP molecule. As more energy is consumed, the ratio of AMP to ATP increases and activates AMP kinase.

When AMP kinase is activated in the periphery, anabolic cellular processes are down-regulated and catabolic cellular processes are up-regulated to generate more ATP [19]. Unlike peripheral tissues, AMP kinase activity in the brain (specifically the hypothalamus) causes more complex reactions. Activation of the AMP kinase in certain areas of the hypothalamus increases food intake and body weight, while suppression of AMP kinase reduces these factors [34]. Male FVB mice (aged 7-9 weeks) were used to observe changes in food intake and body weight through alterations in hypothalamic AMP kinase activity. Mice were implanted with a brain cannula and injected with recombinant adenovirus to express constitutively active AMP kinase (CA-AMPK) or dominant negative AMP kinase (DN-AMPK) in the medial hypothalamus. All mice lost weight two days post injection, but mice expressing DN-AMPK lost significantly more weight than CA-AMPK and control mice and regained weight more slowly. Also, overall food intake in CA-AMPK mice was significantly higher than DN-AMPK and control mice after eight days. This study showed that changes in hypothalamic AMPK activity could affect food intake and body weight. The authors concluded this is effect is partially due to alterations in the expression of orexigenic neuropeptides, neuropeptide Y (NPY), and agouti-related peptide (AgRP). In the same study, the authors discovered that high AMP kinase activity, like those found in CA-AMPK mice, stimulates feeding signals during fasted states. Conversely, low AMP kinase activity, like those found in DN-AMPK mice, suppressed feeding signals during unrestricted feeding. These results provide an insight into how various changes in hypothalamic AMP kinase can influences appetite and food intake.

AMP kinase regulates energy intake and body weight by mediating the opposing effects of anxoregenic (appetite-suppressing) and orexigenic (appetite-stimulating) signals in the hypothalamus. These hypothalamic signals include nutrient/substrate levels (ex. glucose and fatty acids), certain peptides, and circulating hormones [19]. Alterations in AMP kinase activity can have major effects on energy homeostasis in the body and can be seen in those suffering from obesity [22]. A study on male FVB mice was performed to determine whether diet-induced obesity (DIO) impaired AMP kinase response to leptin in the hypothalamus or in muscles [35]. Mice were randomly assigned to the control group (chow diet) or the DIO group (high-fat diet). After 10 weeks on the diets, six mice from each group were injected intraperitoneally with leptin or saline. Food intake after 24 hours of leptin injection in chow fed mice decreased more than 30%, but did not decrease significantly in DIO mice. Leptin-resistance can be found in many cases of obesity, thus alterations in leptin-AMP kinase signaling may play a significant role in appetite control.

Role of Leptin and Ghrelin

AMP kinase activity can be affected by hormonal changes due to fasting and refeeding. Refeeding increases blood glucose and insulin, which triggers a rise in leptin synthesis and secretion. Leptin is classified as an adipokine, a group of peptides and hormones secreted by white adipose tissue, though it is also secreted by the gastric mucosa. The role of gastric leptin is short-lived and is directed more towards food absorption in the intestines, whereas adipocyte leptin is involved feeding signals and energy storage [36]. In the hypothalamus, circulating leptin decreases AMP kinase activity by inhibiting the expression of NYP and AgRP. Under normal conditions, leptin triggers a feedback mechanism to the brain to decrease food intake and

increase energy expenditure [22]. High levels of leptin are seen in obese individuals across all ages due to high percentages in body fat leading to leptin insensitivity. However, calorie restriction has been shown to increase responsiveness to leptin in older rats more so than younger rats [37]. Conversely, very low leptin concentrations have been seen in patients with exercise-induced amenorrhea and anorexia nervosa [38]. Amenorrheic female athletes had significantly lower leptin levels and abnormal diurnal leptin patterns than the weight-match and body-fat matched controls and eumenorrheic athletes. Thus, body fat cannot fully explain the decrease in circulating leptin concentrations. In agreement with this, Weigle et al. discovered a decrease in leptin concentration that was disproportional to body fat loss after a three day (72 hour) fast in male subjects [39].

On the other hand, fasting induces a decrease in blood glucose levels and elicits the secretion of ghrelin, an orexigenic hormone, in the stomach. Ghrelin increases AMP kinase activity mainly in the hypothalamus and in specific tissues – the heart, liver, and adipose tissue. Ghrelin increases the expression of NPY and AgRP, which elicits food intake and fat deposition. In rats, it was indicated that leptin was an upstream regulator of ghrelin secretion, however that cannot be confirmed in human subjects [22]. Unlike leptin, ghrelin is not necessary for maintenance of energy homeostasis. In ghrelin knockout mice, body composition and size, bone density, and food intake were similar to normal control mice [40]. Obese individuals may have an impaired suppression of ghrelin in response to feeding, but plasma concentrations are typically lower than lean individuals [9]. In rats, extreme obesity followed chronic administration of ghrelin in the absence of leptin. Thus, the effects of chronic ghrelin infusion were observed in humans. Continuous infusion of ghrelin increased appetite and food intake in humans in a short-term study [41]. Nine subjects were continually administered ghrelin for 270 minutes. After the

infusion, the subjects were treated to a buffet-style lunch and food intake was recorded. All subjects had an increased food intake and there was no compensatory under-eating the rest of the day.

Some studies have compared the relation of leptin and ghrelin during fasting state. The effects on leptin and ghrelin during normal diurnal conditions, restricted feeding, and sleep deprivation were studied on male Sprague-Dawley rats [20]. The diurnal rhythms of leptin and ghrelin were at opposite times of the day, with peak levels of leptin occurring during active times and peak ghrelin levels occurring during sleep. In the second experiment, 8 rats were put on a 12-hour food restriction diet (RF) for 3 weeks. This was achieved by removal of feeders at dark onset (active time) and returned at light onset (sleep). In a previous study, rats were shown to adapt to this dietary regimen after 1 week. Correspondingly, food intake and body weight were not significantly different between the free feeding rats and the RF rats. High rates of food intake were observed in the first hour of the light period, thus greatly impacting plasma leptin and ghrelin levels. Peak and 24-hour ghrelin levels were significantly higher in the RF rats than the free-feeding group. 24-hour leptin concentrations were significantly higher in the RF rats than control and peak leptin values were also slightly higher, but not significant.

Despite the implications of ghrelin as the “hunger hormone,” few studies have seen any changes in ghrelin concentrations after a period of intermittent fasting. Heilbronn et al. showed decreases in weight and fasting insulin levels in non-obese men and women, but no changes were found in fasting ghrelin levels after 22 days of ADF [9]. Accordingly, no significant changes in plasma ghrelin concentrations were found in 8 males after Ramadan fasting and no association was found between plasma leptin and ghrelin levels [42]. However, leptin levels showed significant reductions during Ramadan for these subjects, which have also been seen in other

studies measuring at leptin concentrations. A few studies showed significant decreases in plasma leptin levels after a period of IF lasting from two weeks to six months [8, 11, 31]. However, one study done in female rats showed no changes in plasma leptin levels after one month of ADF [10]. With such mixed results in humans, observation of ADF on leptin and ghrelin concentrations should be made in order to determine if these factors have a mechanistic role in the positive outcomes of this dietary regimen.

CHAPTER 3

METHODOLOGY

Animals: Fourteen male Wistar rats of similar age and weight (approximately 10 weeks old; 175.07 g ± 6.44) were obtained from Charles River (Wilmington, MA). Rats were randomly assigned to either the ADF group (n=7) or the control group (n=7), and housed individually in a 12-hour light/dark cycle at UNLV's Laboratory Animal Care Facility. Body weight for each rat was recorded prior to the initiation of treatment, once a week during treatment, and prior to euthanization at the completion of the 30-day treatment.

Diet: Standard rodent chow (PicoLab Rodent Diet 20; Lab Diet, St Louis, MO) was given to both groups every morning. The ADF group had alternating 24-hour fasting and feeding days for 30 days. Fasting rats were limited to 25% of estimated daily caloric need on fasting days. About 3 g of regular chow was given initially and then was gradually raised to 6 g as weight increased. This was followed by an ad libitum feeding day, where rats were provided 50-60 g of food. The control group was provided 50-60 g of food everyday for ad libitum feeding for the same 30 days. Both groups had continuous access to water. Food intake for both groups was measured on a digital scale and recorded daily.

Radioimmunoassays (RIA): Blood was obtained via tail clip (0.5 mm tip of tail) from each rat at the end of day 1 (fed), day 2 (fast), day 29 (fed), and day 30 (fast), allowing for changes in hormone levels after a fasting and feeding day to be compared before and after the ADF intervention. All samples were collected between 9:00-10:00am each day. 600µl of blood was collected in capillary tubes (Microvette 600, Sarstedt, Nümbrecht, Germany), centrifuged, plasma removed, and stored at -70° C until assayed. Following the tail clip on days 1 and 2, animals were returned to cages and started on ADF or CON dietary regimen for 30 days. Plasma

samples were analyzed for leptin and ghrelin levels using multi-species leptin RIA kit (EMD Millipore Corporation, Billerica, MA, USA) and rat ghrelin RIA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA), according to manufacturers' instructions.

Blood Glucose: Prior to blood collection in capillary tubes on day 2 and 30, blood glucose levels were determined via blood glucose monitor (Ascensia Contour, Bayer Healthcare LLC, Mishawaka, IN).

Body Composition & Bone Mineral Density: After blood collection on day 30, animals were euthanized via CO_2 inhalation; body composition (percent body fat) and bone mineral density was determined by dual energy x-ray absorptiometry (DEXA) (GE Lunar, Madison, WI) using the small animal software. Animal carcasses were placed on an absorbent underpad to separate the carcass from direct contact with the DEXA surface. Following DEXA scans, epididymal fat pad were removed and weighed, and carcasses were returned to LACF for disposal.

Statistics: Analyses were performed using Statistical Package for the Social Sciences (SPSS, version 23) software. Differences in food intake, body weight, serum leptin, and serum ghrelin before and after treatment were analyzed by mixed model ANOVA. Independent t-test was used to compare mean values of body fat between the ADF and control group. Statistical significance was set to $\alpha=0.05$.

CHAPTER 4

RESULTS

Weight in the control group doubled over the 30-day treatment period (176.6 g; 355.1 g), whereas there was only a 67% increase in the ADF group (173.6 g; 290.3 g) (Table 1). There was a significant interaction between diet regimen and body weight ($F = 41.604$, $p < .001$) (Figure 1). Simple main effects analysis was conducted to determine the nature of the interaction. Independent t-tests showed that there was no significant difference in weight between groups prior to treatment ($p = .405$); however, weight in the ADF group was significantly lower in weeks 1, 2, 3, and 4 ($t = 11.046$, $t = 5.645$, $t = 9.778$, $t = 5.731$, respectively; $p < .001$). One way repeated measures ANOVA showed significant weight gains in both group at each of the time points ($p < .05$), except there was no significant weight gain between Week 2 and Week 3 in the ADF group ($p = .213$).

Daily food intake averaged over 10 days in the ADF was 32% lower than in the control group (Table 2). However, there was no significant interaction found in average daily food intake between the control and the ADF group ($F = 2.127$, $p = .141$) (Figure 2), indicating that food intake increased in both over the 30-day study period as the animals grew. Interestingly, food intake on feeding days in the ADF group was 25% higher than in the control group (Figure 3 and 4). A significant interaction was found between diet regimen and 10-day average intake for fed days only ($F = 3.484$, $p = .047$). Independent t-tests showed the ADF group had significantly higher fed-day intake at each of the 10-day time points ($t = -4.745$, $t = -5.682$, $t = -6.019$, respectively; $p < .001$). One-way repeated measures ANOVA showed significant increases in feeding days for both groups at each of the time points ($p < .05$). This shows that food intake did

increase significantly on feeding days in the ADF group, but the increase was not enough to compensate for the restricted intake on the fasting days.

Blood glucose was significantly lower in the ADF on the last fasting day (Day 30, Post) versus the first fasting day (Day 2, Pre) ($t = 6.087$, $p < .001$). Post ADF blood glucose was also significantly lower than post control blood glucose ($t = 5.162$, $p < .001$) (Figure 3).

Percent body fat and fat pad weight are shown in Table 3. Despite having lower weight gains, the ADF group had significantly higher total percent body fat ($t = -2.848$, $p = .015$) and significantly higher trunk region fat ($t = -2.328$, $p = .038$) than the control group (Figure 4). However, the fat pad weight in the control group was significantly higher than the ADF group ($t = 4.290$, $p = .001$), suggesting a change in fat distribution with ADF. In addition, bone mineral density (BMD) was also significantly decreased in the ADF group ($t = 3.000$, $p = .011$).

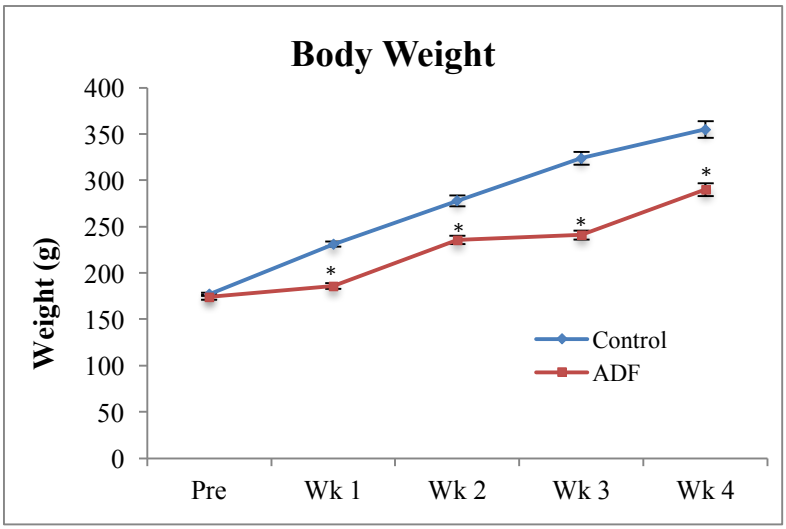
Plasma leptin and ghrelin concentrations are shown in Table 4. Leptin showed no significant interaction with diet regimen ($F = .134$, $p = .895$) (Figure 5). However, there was a change in overall leptin concentration between Day 1 and Day 2 ($p = .007$), and between Day 29 and Day 30 ($p < .001$); this suggests a variation in leptin secretion that is unrelated to diet. Similarly, there was no significant interaction between diet regimen and ghrelin concentrations ($F = 1.256$, $p = .304$) (Figure 6). However, pairwise comparison for ghrelin showed significance between Day 29 and Day 30 ($p = .005$).

Table 1: Body Weight

	Pre	Week 1	Week 2	Week 3	Week 4
Control (g)	176.6 ± 4.89	231.3 ± 7.43	278 ±15.49	324 ± 18.24	355.1 ± 23.33
ADF (g)	173.6 ± 7.79	185.7 ± 7.99*	236.4 ± 11.82*	241 ± 13.10*	290.3 ± 18.77*

Values are mean ± standard deviation; * Significantly different than control ($p < 0.05$)

Figure 1: Body Weight



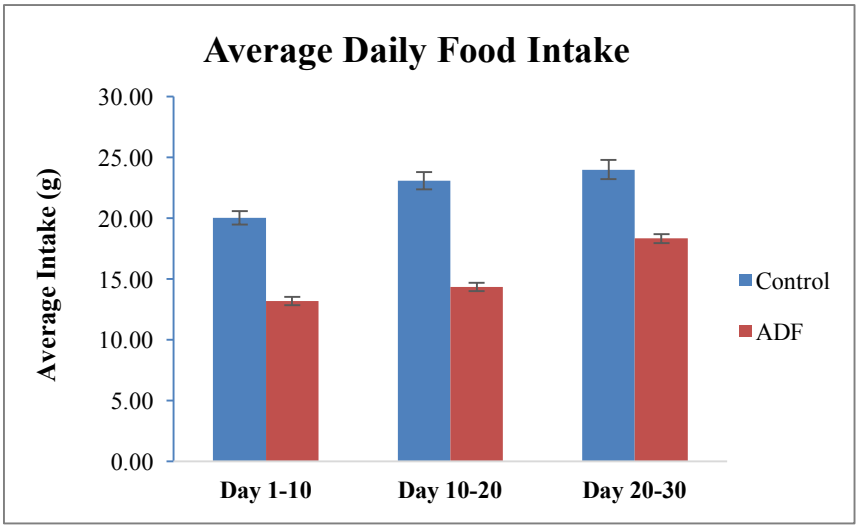
Values are means ± standard error. * Significantly lower than control ($p < 0.05$)

Table 2: 10-Day Average Daily Food Intake

	Day 1-10	Day 10-20	Day 20-30
Control (g)	20.04 ± 1.43	23.10 ± 1.87	24.00 ± 2.03
ADF (g)	13.18 ± 0.92	14.33 ± 0.91	18.32 ± 1.04
Fed Days ADF (g)	24.55 ± 2.07*	28.69 ± 1.81*	30.57 ± 2.05*

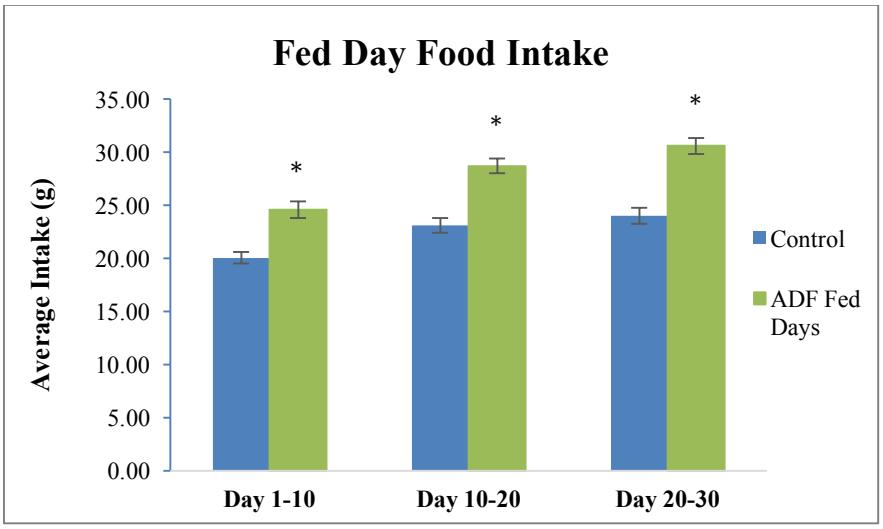
Values are mean ± standard deviation; * Significantly higher than control ($p < 0.001$)

Figure 2: Average Daily Food Intake



Values are means ± standard error.

Figure 3: Average Fed Day Food Intake



Values are mean ± standard error; * Significantly higher than control ($p < 0.001$)

Figure 4: Fed Day Food Intake Timeline

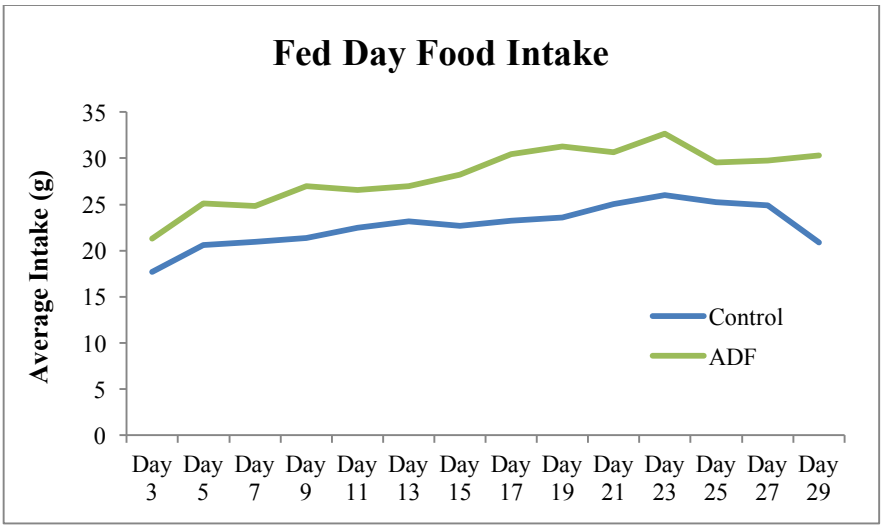
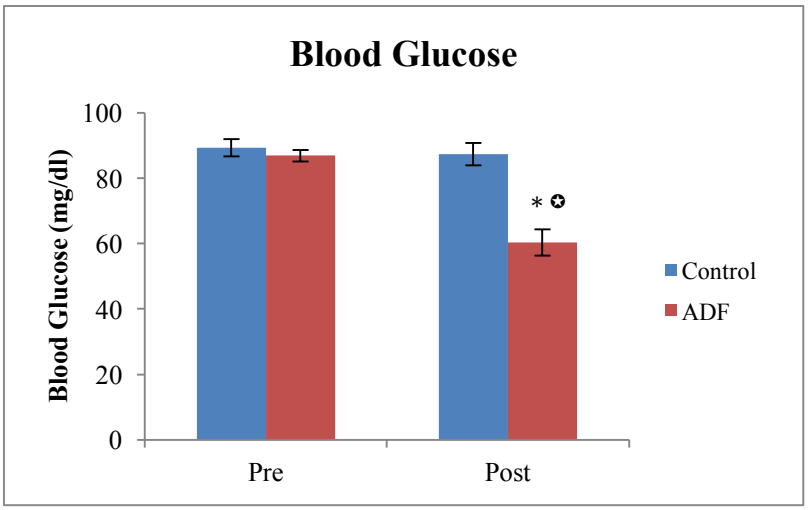


Table 3: Pre & Post-Diet Blood Glucose

	Pre (Day 2)	Post (Day 30)
Control	89 ± 7	87 ± 9
ADF	87 ± 5	60 ± 11*✪

Values are means ± standard deviation; * Significantly different from Pre value ($p < 0.001$); ✪ Significantly different from control ($p < 0.001$)

Figure 5: Pre & Post-Diet Blood Glucose



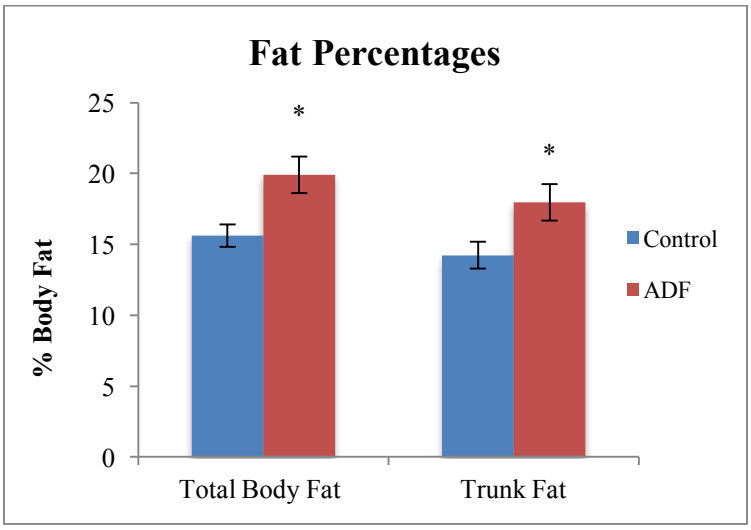
Values are means ± standard error. Measured following the first fasting day (Pre) and the last fasting day (Post); * Significantly different from Pre value ($p < 0.001$); ✪ Significantly different from control ($p < 0.001$)

Table 4: Body Composition Results

	Body Fat %	Trunk Fat %	Fat Pad (g)
Control	15.6 ± 2.09	14.3 ± 2.51	3.57 ± 0.41*
ADF	19.9 ± 3.41*	18.0 ± 3.45*	2.76 ± 0.29

Values are mean ± standard deviation; * Significantly higher value ($p < 0.05$)

Figure 6: Body Fat Percentage



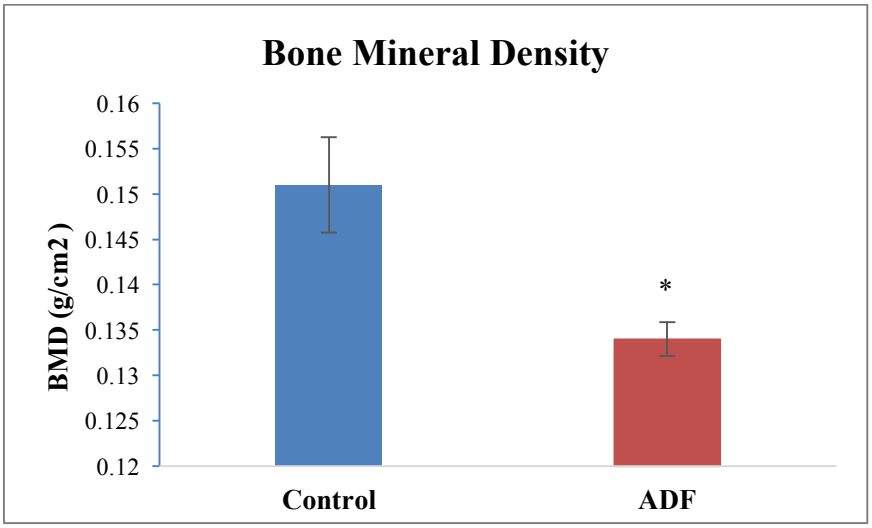
Values are mean ± standard error; * Significantly different from control ($p < 0.05$)

Table 5: Bone Mineral Density

	BMD (g/cm^2)
Control	0.151 ± 0.014
ADF	0.134 ± 0.005*

Values are mean ± standard deviation; * Significantly lower value (p = .01)

Figure 7: Bone Mineral Density



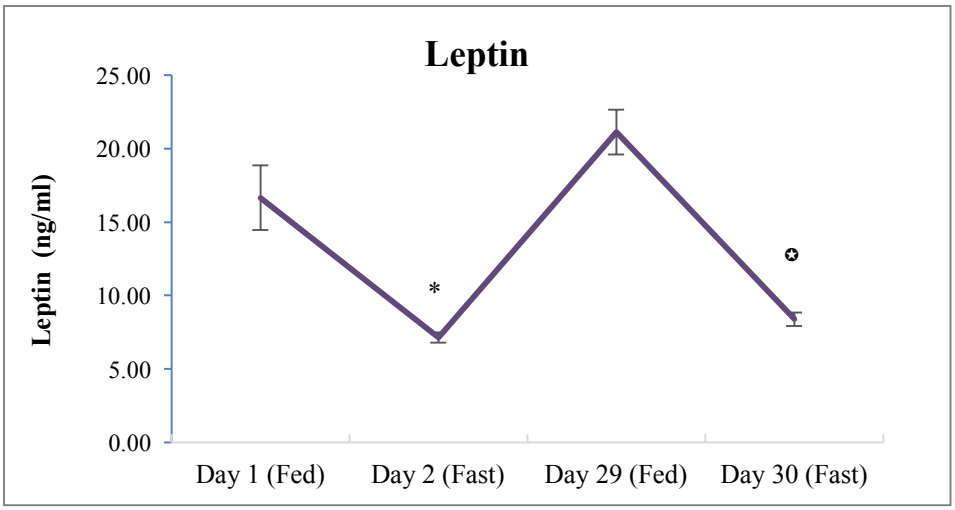
Values are mean ± standard error; * Significantly lower from control (p = .01)

Table 6: Group Means for Plasma Leptin and Ghrelin

		Day 1 (Fed)	Day 2 (Fast)	Day 29 (Fed)	Day 30 (Fast)
Leptin (ng/ml):	**Control (n=7)**	15.95 ± 10.0	7.53 ± 1.47	20.42 ± 5.25	8.15 ± 1.09
	ADF (n=7)	17.36 ± 6.73	6.78 ± 1.05	21.85 ± 6.43	8.63 ± 2.25
	Total (n=14)	16.66 ± 8.24	7.15 ± 1.29*	21.14 ± 5.69	8.39 ± 1.72✪
Ghrelin (pg/ml):	**Control (n=7)**	1170.7 ± 576.1	1193.0 ± 419.0	922.7 ± 249.6	1196.1 ± 248.4
	ADF (n=7)	1085.9 ± 229.6	1306.5 ± 361.5	854.8 ± 251.9	1475.3 ± 304.5
	Total (n=14)	1128.3 ± 423.6	1249.8 ± 380.5	888.8 ± 243.5	1335.7 ± 303.7✪

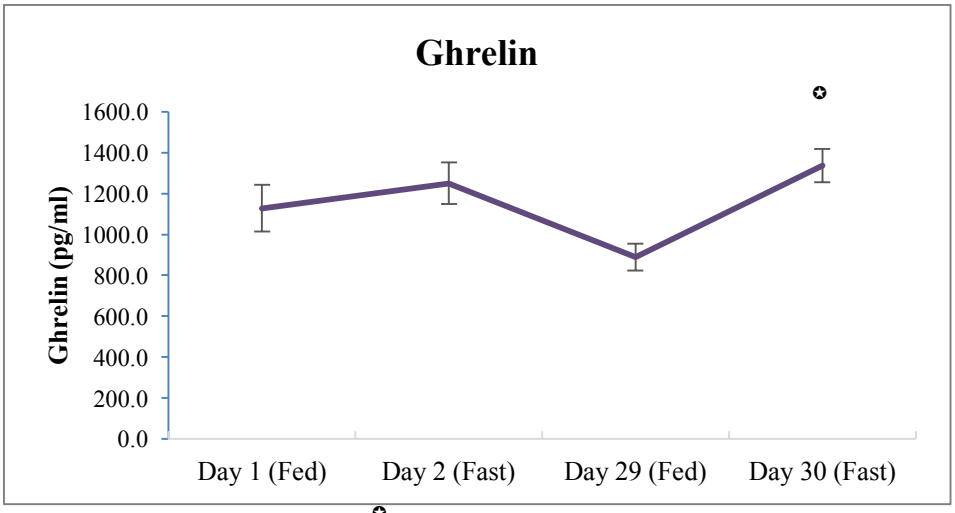
Values are mean ± standard deviation; * Significantly different from Day 1 (p = 0.007); ✪ Significantly different from Day 29 (p < 0.005)

Figure 8: Leptin Concentrations



Values are mean ± standard error; * Significantly different from Day 1 ($p = 0.007$); ✪ Significantly different from Day 29 ($p < 0.001$)

Figure 9: Ghrelin Concentrations



Values are mean ± standard error; ✪ Significantly different from Day 29 ($p = 0.005$)

CHAPTER 5

DISCUSSION

Alternate-day fasting has become a popular dieting method because it promised weight loss along with the ability to eat unrestricted during the 24-hour feeding days, despite very low caloric intakes sustained on fasting days. The main concept of the the diet claims that individuals cannot consume over double their daily need on feeding days, which leads to overall calorie deficiencies and weight loss over time [1, 2]. Because rodents would not be influenced by psychological appetite triggers, we expected food consumption on feeding days to increase enough to offset the caloric deficits on restricted feeding days. While food consumption did increase on feeding days, it was not enough to compensate for the caloric deficit incurred on fasting days. Food intake in ADF rats only increased by 20% (or 5 g) on feeding days when compared to the controls. Consequently, weight gain was stunted in the ADF group. It's possible that the rodents were not able to tolerate more food in the 24-hour period, limiting food intake on feeding days. Previous research has shown that when treating for malnourishment, food must be re-introduced slowly to avoid complications caused by rapid imbalances of fluid and electrolytes, known as refeeding syndrome [43]. Research on refeeding syndrome shows that there is a limited amount of food that the body can process after a period of deprivation without adverse effects. Though refeeding syndrome is generally seen after prolonged starvation or malnutrition, this condition may provide insight into why food intake was lower than expected on fed days.

Plasma leptin and ghrelin concentrations were measured to determine if the increase in food intake could be linked to physiological changes. In both human and rodent studies, continuously infused exogenous ghrelin led to increased food intake, with the greatest effect seen during central administration [22, 41, 44-46]. However, the role of endogenous ghrelin is still

controversial. Some studies have shown that in ghrelin knockout mice, food intake and appetite were similar to wild-type counterparts [40, 47]. However, other studies have shown the importance of endogenous ghrelin at meal initiation [46, 48, 49]. With of this in mind, we hypothesized plasma ghrelin concentrations would not change, since we did not expect total intake to be different between groups. Although there was a hyperphagic response on feeding days in the ADF group, there were no significant differences in ghrelin between the ADF and control group after 30 days. The possibility of increased ghrelin sensitivity in the ADF rats cannot be ruled out. Increased ghrelin sensitivity may contribute to the increase in food intake after fasting days, and would also account for the higher percent body fat in the ADF group since ghrelin has been shown to increase adiposity in rodents [49, 50]. Recent evidence has shown that ghrelin promotes fat storage in white adipose tissue and decreases fat utilization in skeletal muscles, while increasing carbohydrate utilization [46, 47, 49]. Similar to other rodent studies with ADF treatments [15-17, 51], fasting blood glucose concentration decreased in the ADF group after 30 days and was significantly lower than the control group. This suggests a metabolic shift in fuel usage in the ADF group. Under normal conditions, the reciprocal relationship between fat and carbohydrate utilization is necessary to maintain the energy needs of various tissues without having to constantly eat. However, alterations in food availability can disrupt energy partitioning, leading to excess storage or expenditure [52]. How animals use energy can generally be partitioned into two main components: maintenance/repair and production/growth. Since food was removed regularly in the ADF group, a shift in substrate utilization may have been necessary to store available energy during feeding days for metabolic maintenance and repair on fasting days. Evidence for this can be seen with reduced rate of

growth and decreased bone mineral density in the ADF group, plus the increased total body and trunk fat relative to the control group.

The expectation of the alternate day fasting diet is that fat mass would decrease, because the long fasting intervals would trigger an increase in lipolysis and fat utilization for fuel, as has been shown in previous studies [2, 9, 11]. However, we observed the exact opposite. Even though weight gain was less in the ADF group compared with the control group, percent total body fat was significantly higher with the feeding intervention (Table 3). Trunk fat percentage was greater in the ADF group compared with the control group. However, epididymal fat pad weight was greater in the control animals, indicating an altered pattern in fat distribution in response to ADF. These results are in keeping with the finding of a redistribution of visceral fat to subcutaneous fat in mice with calorie restriction and ADF [10]. One explanation for this response is that the restriction in food intake was sufficient enough to trigger the starvation response leading to increased fat storage. Fat is the body's long-term storage form of energy, and it is thought that this is a mechanism inherited from the hunter-gatherer lifestyle of our ancestors. As stated above, the ability to switch between fat and carbohydrate use allows for some flexibility in diet and meal timing. When food was not always readily available, extra energy was converted into fat during times of food abundance to compensate for intermittent food shortages [53]; the "thrifty gene hypothesis." Though this theory is still strongly debated, our results support this concept. It is possible that fasting on alternating days could have stimulated the genes that promote fat storage in the ADF group. In contrast, the control group had food that was constantly available favoring energy use for growth, as indicated by their higher body weight, bone mineral density, and lean body mass percentage.

Unlike ghrelin, high levels of leptin stimulate energy expenditure and decrease food intake [20, 37, 54]. In rodents, exogenous leptin administration induces weight loss and decreased fat mass by activating lipid oxidation and apoptosis in adipocytes [55]. Leptin, which is primarily secreted from adipose tissue, increases after feeding in response to high levels of insulin and certain amino acids and decreases in response to fasting. Unfortunately, peripheral administration of leptin did not result in weight loss for most obese individuals [36, 38, 55]. Additionally, elevated leptin levels are found in many obese individuals due to higher amounts of body fat and overexpression of leptin from adipose tissue [36]. The inability of these high leptin concentrations to decrease food intake and induce weight loss in these individuals indicates a defect in leptin receptors and/or post-receptor signaling [55, 56]. The prevailing belief is that the physiological role of leptin is to signal the brain during states of negative energy balance and decreased energy stores. Lower levels of leptin appear to greatly stimulate the mechanisms for fat storage when percent body fat is low. When percent body fat is high, there appears to be a mechanistic defect in leptin response and signaling, thus no changes in body fat are observed [53].

During starvation, leptin levels decrease rapidly to signal the brain for adaptive metabolic changes [55, 57]. Because of this, we expected leptin levels to be lower in the ADF group, especially on fasting days, with respect to the controls. Our results showed no differences in plasma leptin concentrations between diet regimens. Leptin levels in the ADF group were high after feeding and low after food restriction. This is consistent with a normal diurnal variation leptin. However, the finding of a similar pattern of secretion in the freely eating control group is suggestive of a circadian rhythm in leptin secretion, rather than a normal feeding-induced diurnal

pattern [20, 58, 59]. This novel finding has not been reported in any other studies to our knowledge, as measurements of leptin on consecutive days with ADF have not been reported.

Our findings indicate that alternate day fasting could be a viable method for weight loss as food intake does not appear to increase significantly on feeding days, but conversely may result in increases in body fat. These effects may be related to an increased sensitivity to circulating ghrelin but are unrelated to changes in leptin. Alternate day fasting appears to have triggered an adaptive response which led to a shift in the use of energy from growth/production to body maintenance and repair. This response is in keeping with alterations in substrate utilization, evidenced by an increased percent body fat with ADF. In addition, the blood glucose in ADF animals on a restricted feeding day at the end of the study was significantly decreased compared with control animals, indicating a greater utilization of carbohydrates for energy. With the continuous search for new and effective weight loss strategies, more research is necessary to fully understand the complex metabolic responses to dieting and intermittent fasting. The physiological factors that influence food intake and body composition is just one element of energy homeostasis. However, the ability to manipulate these factors could lead to the development of treatment options for both obesity and anorexia/cachexia.

In the present study, male Wistar rats were used, however it not clear if female rats would response similarly. Female rats have been shown to preserve lean mass to a greater degree than male counterparts during food restriction protocols [60]. As exercise is known to spare lean body mass, future research on the effects on body composition and hormone levels with the inclusion of an exercise regimen with ADF is warranted. Additionally unlike males, female Wistar rats have been shown to increase food intake in response to exercise [60]. Though our rats were developmentally considered adults at 10 weeks of age, the control group demonstrated that

they were still growing, thus older rodents may generate differing results. Older, weight-stable rodents may have different metabolic and hormonal responses to the ADF regimen. The stability of the effects of body composition could be determined by switching the ADF group back to an ad libitum regimen. Finally, substituting a high-fat chow diet (similar composition to Western diets) for the standard chow that was used in the present study may provide more insight into the metabolic changes that can occur in human on an ADF regimen.

APPENDIX I:

IACUC APPROVAL & PROTOCOL

UNLV IACUC

Approved

DATE:	October 12, 2015
TO:	John Young
FROM:	UNLV IACUC
PROJECT TITLE:	[797477-2] The Effects of Alternate Day Fasting on Appetite-related Hormones in Rats
REFERENCE #:	
SUBMISSION TYPE:	Revision
ACTION:	APPROVED
DECISION DATE:	October 12, 2015
EXPIRATION DATE:	October 11, 2018

Thank you for your submission of Revision materials for this research project. The UNLV IACUC has APPROVED your submission. All research must be conducted in accordance with this approved submission.

Please note that any revision to previously approved materials must be approved by this committee prior to initiation. Please use the appropriate amendment form for this procedure.

Please report all NON-COMPLIANCE issues regarding this project to this committee.

Annual Review

This project requires annual review by this office. Please use the appropriate annual review form for this procedure.

Renewals

All projects expire three years after the approval date. You must submit and obtain approval for a new project prior to October 11, 2018 to avoid any lapse in approval.

If you have any questions, please contact Kevin Bergeron at (702) 895-5453 or kevin.bergeron@unlv.edu. Please include your project title and reference number in all correspondence with this committee.

Office of Research Integrity - IACUC & IBC
4505 Maryland Parkway . Box 454022 . Las Vegas, Nevada 89154-4022
(702) 895-5453

UNLV **Institutional Animal Care and Use Committee**

PROTOCOL FOR ANIMAL CARE AND USE

1. PROJECT OR EXERCISE TITLE: The Effects of Alternate Day Fasting on Appetite-related Hormones in Rats

2. PRINCIPAL INVESTIGATOR/COURSE DIRECTOR:
NAME and TITLE: Dr. John Young
DEPARTMENT: Kinesiology and Nutrtion Sciences
OFFICE PHONE: 702-895-4626 EMERGENCY PHONE: 702-682-7792
LAB CONTACT (TECHNICIAN) and PHONE NUMBER:
LOCATION (BUILDING/LAB#):
E-MAIL:john.young@unlv.edu FAX: 5-1500
Does the PI have direct contact with the animals? ☒ Yes ☐ No

3. LIST RESEARCHERS AND STAFF INVOLVED WITH ANY PROTOCOL PROCEDURES:

Name	Responsibility	Office Phone	Emergency Phone
Debra Tacad	Student	702-895-4875	702-242-2124
Dr. Laura Kruskall	Co-PI	702-895-4985	702-274-0370
Dr. Richard Tandy	Data Analysis		
Dr. James Navalta	Data Analysis	702-895-2344	

Hit tab to add more rows to any of the tables in this form. Use the down arrow to move out of the table to the next field.

4. TRAINING FOR ALL PERSONNEL WORKING WITH ANIMALS ON THIS PROTOCOL:

Name	Role in this protocol (PI, co-PI, student, etc.)	Experience working with species	Training Received
Jack Young	PI	38 Years	1991 UNLV
Laura Kruskall	Co-PI	13 years	2001 UNLV
Debra Tacad	Student	1 year	2015 UNLV

5. PROTOCOL STATUS: ☒ New/Renewal ☐ Modification

START DATE: 8/2015 ANTICIPATED COMPLETION DATE (3 yr max): 11/2015

6. FUNDING SOURCE: ☒ DEPARTMENT ☒ OTHER INTRAMURAL SOURCE
☐ EXTRAMURAL SOURCE SPECIFY SOURCE:
IF FUNDED, GRANT NUMBER:

7. CATEGORY OF ETHICAL CONCERN APPLICABLE TO THIS PROTOCOL (see Appendix A): C

8. JUSTIFICATION: THIS SECTION IS REQUIRED FOR ANY PROJECT IN WHICH THERE IS A POTENTIAL FOR PAIN, DISTRESS, OR DISCOMFORT THAT **CANNOT BE ALLEVIATED**. EXAMPLES OF SUCH PROJECTS INCLUDE INDUCED DISEASE STATES WHICH CAUSE SEVERE SYMPTOMS OR DEATH, SURGICAL STUDIES RESULTING IN SEVERE POST-OPERATIVE DISCOMFORT OR LOSS OF FUNCTION, USE OF SEVERE AND UNAVOIDABLE NOXIOUS STIMULI, AND SO FORTH.

a. Will there be unrelieved pain or stress (defined as lasting for more than a moment, i.e. longer duration or more painful than a needlestick)? ☒ No ☐ Yes

b. If yes, provide justification and an indication of the number of animals, per year, that are going to experience unrelieved pain or stress must be provided.

c. Is death an end-point? ☒ No ☐ Yes

d. If yes, explain why some earlier end-point data cannot be used instead (provide objective supporting data).

9. WILL THE PROJECT OR EXERCISE INVOLVE EXPOSURE OF ANIMALS OR ANIMAL HANDLERS TO:

BIOHAZARDS?	☒ NO	☐ YES	IF YES, SPECIFY:
RADIOISOTOPES?	☒ NO	☐ YES	IF YES, SPECIFY:
CARCINOGENS?	☒ NO	☐ YES	IF YES, SPECIFY:
TOXIC CHEMICALS?	☒ NO	☐ YES	IF YES, SPECIFY:

IF ANY ITEM ANSWERED YES, PLEASE COMPLETE AND **ATTACH SUPPLEMENT FORM 1.** An approved Institutional Biosafety Committee protocol may be required.

10. ITEMS WHICH APPLY TO THIS PROJECT:

☒ Blood and/or tissue collection	☐ Survival surgery
☐ Antibody production and collection	☐ Non-survival surgery
☐ Behavioral studies	☐ Aseptic surgery
☐ Prolonged physical restraint	☐ Multiple surgeries on the same animal
☒ Food or water deprivation	☐ Alleviated pain
☐ Environmental extremes	☐ Unalleviated pain
☐ Electrical stimuli	☐ Anesthetics used
☐ Induction of trauma	☐ Single housing
☐ Work to be done off campus	☐ Immobilizing agents without anesthesia
☐ Field studies	

11. IS SURGERY INVOLVED IN THE PROPOSED PROJECT?
☒ No ☐ Yes IF YES, **ATTACH SUPPLEMENT 2**

12. ARE STRESSFUL OR PAINFUL PROCEDURES, OTHER THAN SURGERY, PART OF THIS PROJECT?
☒ No ☐ Yes IF YES, **ATTACH SUPPLEMENT FORM 3**

13. ARE PROLONGED (MORE THAN A FEW HOURS) PHYSICAL RESTRAINT PROCEDURES PART OF THIS PROJECT?
☒ No ☐ Yes IF YES, **ATTACH SUPPLEMENT FORM 4**

14. WILL GENETICALLY ENGINEERED OR OTHER MUTANT ANIMALS BE PRODUCED OR MAINTAINED THAT MAY BE EXPECTED TO DEVELOP CLINICAL SIGNS AS A RESULT OF THE MUTATION?
☒ No ☐ Yes IF YES, **ATTACH SUPPLEMENT FORM 5. AN APPROVED INSTITUTIONAL BIOSAFETY COMMITTEE PROTOCOL IS ALSO REQUIRED.**

15. WILL TISSUES, CELLS OR OTHER PRODUCTS DERIVED FROM ANIMALS BE SHARED OR A TISSUE BANK ESTABLISHED FOR FUTURE SHARING WITH OTHER INVESTIGATORS DURING OR AFTER THE PROJECT?
☒ No ☐ Yes

16. ARE BEHAVIORAL STUDIES INVOLVED IN THIS PROJECT? ☐ Yes ☒ No
Do any of the following procedures or conditions apply?

Food Reward	☐	
Electrical Shock	☐	Strength and duration:
Food Deprivation	☒	Kcal/day provided: 10-15 kcal/every other day for fasting days
Water Deprivation	☐	ml/kg provided:

If a conditioning protocol applies, please explain the purpose.

Criteria for monitoring the condition of the animals during food and water deprivation:

For a 150 gm rat, daily needs per rat are approximately 15 gm/day of rat chow or 60 kcal/day (at 4 kcal/gm). Alternate day fasting regimen requires a restriction of 20-25% of daily needs on fasting days, which equals to 3-4 gm or 10-15 kcals every other day followed by an ad libitum feeding the next day. Both groups will have continuous access to water.

1. Baker, H.J., Lindsey, R. and Weisbroth S.H. Selected Normative Data. The Laboratory Rat, Vol. 1. Academic Press, 1979

2. Antoni, R., L. Johnston, K., L. Collins, A., & Robertson, M. D. (2014). The Effects of Intermittent Energy Restriction on Indices of Cardiometabolic Health. *Research in Endocrinology*, 1-24. doi: 10.5171/2014.459119
3. Varady, K. A., Allister, C. A., Roohk, D. J., & Hellerstein, M. K. (2010). Improvements in body fat distribution and circulating adiponectin by alternate-day fasting versus calorie restriction. *The Journal of Nutritional Biochemistry, 21*(3), 188-195. doi: http://dx.doi.org/10.1016/j.jnutbio.2008.11.001
4. Varady, K. A., Hudak, C. S., & Hellerstein, M. K. (2009) Modified alternate-day fasting and cardioprotection: relation to adipose tissue dynamics and dietary fat intake. *Metabolism - Clinical and Experimental, 58*(6), 803-811. doi: 10.1016/j.metabol.2009.01.018
5. Varady, K. A., Roohk, D. J., Loe, Y. C., McEvoy-Hein, B. K., & Hellerstein, M. K. (2007). Effects of modified alternate-day fasting regimens on adipocyte size, triglyceride metabolism, and plasma adiponectin levels in mice. *Journal of Lipid Research, 48*(10), 2212-2219. doi: 10.1194/jlr.M700223-JLR200

17. ARE THERE ANY EXCEPTIONS TO THE GUIDE (*Guide for the Care and Use of Laboratory Animals, National Research Council*)? ☐ Yes ☒ No

If yes, describe the exceptions in detail including the scientific justification for the exception. The most common exceptions include; single housing, lighting, food/water variations, cage changes, temperature, ventilation, or euthanasia method that differs from the guide.

18. ANIMAL CENSUS AND HOUSING - Use one line/species/pain category. Repeat table rows as needed.

Common Species Name/Scientific Name	Strain/Stock Breed	Sex	Starting Age/Weight	Total # Requested	Pain Category
Rat/ Rattus norvegicus	Wistar/Sprague-Dawley	Male	140-160 g (10 weeks of age) Puberty is reached at 50 days ±10 days. 10 weeks ensures no changes in weight related to puberty rather than treatment.	14	N/A

During the course of the study, list the source of the animals and the expected average daily census:

Average Daily Census: 12-14	Source of the animals: Approved vendor

Will these animals be bred? If so explain, please include numbers of animals bred and anticipated number of offspring.

No.

19. LOCATION OF ANIMAL HOUSING AND USE AREAS:

ANIMAL HOUSING FACILITY: UNLV Lab Animal Care Facility
WILL ANIMALS BE REMOVED FROM THE HOUSING FACILITY FOR USE ELSEWHERE?
☐ No ☒ Yes IF YES, SPECIFY LOCATIONS:

Procedures	Building or Site	Room
Non surgical procedures or conditions	LACF	
Nonsurvival surgery	N/A	
Survival surgery	N/A	
Postsurgical/Postanesthesia/Postprocedural Recovery	N/A	

Maximum number of hours at one time the animals will be kept in the laboratory/surgery:
☒ 0-12 hrs ☐ 12-24 hrs ☐ over 24 hrs *(Animals kept over 24hours require an establishment of an IACUC approved satellite facility.)*

20. ARE THERE ANY SPECIAL REQUIREMENTS FOR ANIMAL HOUSING, DIETS, RESTRAINT, OR PROCEDURES FOR DISPOSAL? ☒ Yes ☐ No

IF NO, ANIMALS WILL BE MAINTAINED ACCORDING TO THE STANDARD OPERATING PROCEDURES. IF YES, PROVIDE (AN) EXPLANATION(S) FOR EACH PARAMETER INVOLVED (E.G., LIGHT:DARK CYCLES, HUMIDITY, AMBIENT TEMPERATURE, CAGING BEDDING, DIETS, AND SO FORTH):

12 hr light/dark cycle, continuous access to water, standard chow diet provided by UNLV LACF, alternate day fasting group (n=7) will be given food ad libitum during feeding days and then fasted every other day (given 20-25% of daily needs).

1. Varady, K. A., Roohk, D. J., Loe, Y. C., McEvoy-Hein, B. K., & Hellerstein, M. K. (2007). Effects of modified alternate-day fasting regimens on adipocyte size, triglyceride metabolism, and plasma adiponectin levels in mice. *Journal of Lipid Research, 48*(10), 2212-2219. doi: 10.1194/jlr.M700223-JLR200
2. Wan, R., Camandola, S., & Mattson, M. P. (2003a). Intermittent fasting and dietary supplementation with 2-deoxy-D-glucose improve functional and metabolic cardiovascular risk factors in rats. *The FASEB Journal*. doi: 10.1096/fj.02-0996fje

21. INSTRUCTIONS FOR DISPOSITION OF SICK OR INJURED ANIMALS

Call Investigator ☐ Veterinarian (or Designee) to Treat ☐ Euthanize ☒

22. INSTRUCTIONS FOR DISPOSITION OF DEAD ANIMALS

Call Investigator ☐ Veterinarian (or Designee) to Necropsy ☐ Bag for Disposal ☒

23. IF WILD OR EXOTIC SPECIES ARE TO BE USED:

a. ARE SPECIAL PERMITS REQUIRED? ☐ Yes ☒ No
IF YES, ATTACH A COPY OF APPLICABLE PERMITS OR PERMIT APPLICATIONS

b. WILL ANIMALS BE OBSERVED, MANIPULATED, OR TRAPPED IN THE WILD? ☐ Yes ☒ No
IF YES, AND THEY HAVE NOT ALREADY BEEN ADDRESSED, DESCRIBE THE FIELD PROCEDURE(S) INCLUDING THE GEOGRAPHIC LOCATION(S) INVOLVED:

24. ADMINISTRATION OF ANESTHETICS AND ANALGESICS.

Species	Drug	Route	Site/location	Volume	Dosage	Frequency

25. ADMINISTRATION OF EXOGENOUS SUBSTANCES

Complete this section for all drugs, infectious agents, carcinogens, toxins, experimental compounds, etc.

Species	Drug/Substances	Route	Site/Location	Volume	Dosage	Frequency

All substances must be pharmaceutical grade. If non-pharmaceutical grade substances will be used, please list the substance and explain why a pharmaceutical grade cannot be used:

Other Comments:

26. ANTEMORTUM FLUID/TISSUE COLLECTION

Species	Type of Fluid or Tissue	Volume or Mass	Frequency of Collection	Method or Route of Collection	Anesthesia or Sedation Used?
Rat	Blood samples	300 µl	Day 1, 2, 29 and 30	Tail bleeding	No

27. EUTHANASIA

Species	Method and Secondary Method (if applicable)	Drug (if applicable)	Dose of Drug mg/kg (if applicable)	Route
Rat	CO_2			inhalation
Rat	Bilateral pneumothorax			

	Note: carcasses must be intact for DEXA scans, so opening chest cavity and severing heart will be done after the DEXA scan is completed			

28. LAY SUMMARY

This summary may be forwarded to the Office of Public Affairs. They may use it to describe your research to the media. The lay summary should be brief and readily understandable to the general public. Provide a summary of the project that explains the need for the research, what the project goals are, and how the use of animals will help reach the stated goals:

Appetite control is one of the harder obstacles to overcome when trying to lose weight. Recently, alternate-day fasting (ADF) has grown in popularity as an alternative to continuous calorie restriction (CCR) diets as a method for improving health and controlling appetite. One possible explanation for this effect could be due to a regulation of appetite hormones, such as leptin and ghrelin. Though several ADF studies have been done with human subjects, this study will focus on appetite-related hormone response in rats. Animal models provide an appropriate form of treatment as animal studies reduce environmental factors that may affect results. Thus, the purpose of this study is to examine the effects of a 30-day ADF trial on appetite and food intake via changes in body weight and levels of leptin and ghrelin in rats. METHODS: 10 week old Wister/Sprague-Dawley albino rats will be housed individually at UNLV's LACF. Animals will be randomized into one of two groups the ADF group (n=7) and the control group (n=7). Both groups will have continuous access to water. The ADF group will have alternating 24-hour fasting and feeding days for 30 days. Fasting rats will be limited to 1 pellet each (about 3-4g) of regular chow on fasting days and will be given food ad libitum (about 30-40g) on feeding days. The control group will have food ad libitum everyday for the same 30 days. Food intake and body weight for both groups will be measured on a digital scale and recorded daily. 300 µl blood samples will be collected into micro capillary tubes via tail clip on day 1, day 2, day 29, and day 30, allowing for changes in hormone levels after a fasting and feeding day to be compared before and after the ADF intervention. Sample tubes will be centrifuged; plasma will be removed and stored for leptin and ghrelin assay. Following euthanasia, assessments of lean body mass and fat mass will be determined from weighing and the use of dual energy x-ray absorptiometry with small animal software. After DEXA scan, epididymal fat pads will be removed and weighed.

29. EXPERIMENTAL SUMMARY

ALL 3 QUESTIONS MUST BE ANSWERED.

a. What are the objectives or underlying hypotheses of the experiment?

Weight loss continues to be a growing concern for many people in industrialized countries. Key components in weight loss are increases in daily physical activity level and diet modifications including appetite control. Though appetite and hunger are sometimes used interchangeably, there are distinct differences between the two. Hunger is defined as the physiological need for food due to depletion of nutrients and is associated with discomfort or weakness caused by lack of food. Appetite is the desire to eat food, which can be separate from hunger, and is influenced by the external factors such as the smell of food being cooked, food advertisements, social settings, etc.

Intermittent fasting (IF) has grown in popularity as an alternative to the more common diet method of calorie restriction (CR) for improving health and controlling appetite. Many deviations of IF exist, however alternate-day fasting (ADF) has become a common variation that has been studied in the past decade. In ADF, food is completely removed or extremely reduced (less than 500 kcals/day) for 24 hours on alternating days, which is followed by a non-fasting day where food is consumed *ad libitum*. The ability to eat whatever you want (on feeding days) without having to count calories becomes a very attractive idea for most people. The main assumption of this diet is that most people cannot consume twice their caloric need on the feeding days, thus lowering caloric intake over a period of time. Though fasting for 24 hours seems difficult, advocates of the diet say that their desire to eat lessens on fasting days, and studies have shown high compliance during short-term ADF trials (Krista A Varady, Bhutani, Church, & Klempel, 2009). The objective of this study is to determine whether the anecdotal results ADF diets are praised for can be attributed to hormonal/metabolic changes within the body.

The purpose of this study is to examine the effects of a 30-day ADF trial on appetite and food intake via changes in body weight and levels of leptin and ghrelin in rats. We hypothesize that there will be a significant difference in leptin and ghrelin levels between the ADF and control group, along with significant decrease in body weight and food intake in the ADF group. ADF has been shown to promote weight loss, extend lifespan, and protect against insulin disorders, cardiovascular disease, and neurological disorders in mice (Stote et al., 2007). Few studies have examined the effects of ADF on the appetite-related hormones leptin and ghrelin in relation to chronic diseases such as diabetes and cardiovascular disease. However, no studies have examined the possible interactions of these hormones and its specific relation to food intake.

b. What happens to the animals from start to finish (please give a detailed description of all procedures)?

(N=14) Sprague-Dawley rats will be obtained from approved vendor. Rats will be housed individually in a 12 hr light/dark cycle in UNLV's Lab Animal Care Facility (LACF). Animals will be randomized into one of two groups, the alternate-day fasting group (ADF) or the control group (CON). Water will be available ad libitum to both groups. Animals will be weighed and blood will be collected by tail clip (0.5 mm tip of tail) on fasting and feeding days before and after dietary treatment, which will fall on day 1, day 2, day 29, and day 30. Blood samples to measure leptin and ghrelin (300 µl) will be collected in micro capillary tubes, centrifuged, plasma removed, and stored at -60 C until assayed. Following the tail clip, animals will be returned to cages and started on ADF or CON dietary regimen for 30 days. Body weight and food intake will be measured daily before feeding. After blood collection on day 30, animals will be euthanized via CO_2 inhalation, animals will be transported to BHS 335 where body composition will be determined by dual energy x-ray absorptiometry (DEXA) with small animal software. Animal carcasses will be placed on an absorbent underpad separating the carcass from direct contact with the DEXA surface. Following DEXA scans, After DEXA scan, epididymal fat pads will be removed and weighed, and carcasses will be returned to LACF for disposal.

c. Why was this species or strain selected?

When examining hormonal changes due to dietary modifications, it is important to protect the subjects from outside factors that may affect the results. Diet compliance of the subjects is a main issue in experiments with dietary treatments and is very difficult to control in human without 24-hour supervision. Even with attempts to educate subjects on diet parameters, it is still possible that subjects will consume too much food during fasting days. Also, food intake during feeding days could be inaccurately recorded by subjects, which will affect results. Physical activity is another factor that can affect leptin and ghrelin levels, but is difficult to control in human subjects. Other research examining the effects of alternate day fasting has relied on animal models, specifically rats. Previous research focusing on the relationship between alternate day fasting and hormonal changes have used a rat model to limit the influence of extraneous variables on the results. [1-6]

1. Castello, L., Froio, T., Maina, M., Cavallini, G., Biasi, F., Leonarduzzi, G., . . . Chiarpotto, E. (2010). Alternate-day fasting protects the rat heart against age-induced inflammation and fibrosis by inhibiting oxidative damage and NF-kB activation. *Free Radical Biology and Medicine, 48*(1), 47-54. doi: http://dx.doi.org/10.1016/j.freeradbiomed.2009.10.003
2. Varady, K. A., Allister, C. A., Roohk, D. J., & Hellerstein, M. K. (2010). Improvements in body fat distribution and circulating adiponectin by alternate-day fasting versus calorie restriction. *The Journal of Nutritional Biochemistry, 21*(3), 188-195. doi: http://dx.doi.org/10.1016/j.jnutbio.2008.11.001
3. Varady, K. A., & Hellerstein, M. K. (2007). Alternate-day fasting and chronic disease prevention: a review of human and animal trials. *The American Journal of Clinical Nutrition, 86*(1), 7-13.
4. Varady, K. A., Hudak, C. S., & Hellerstein, M. K. (2009). Modified alternate-day fasting and cardioprotection: relation to adipose tissue dynamics and dietary fat intake. *Metabolism - Clinical and Experimental, 58*(6), 803-811. doi: 10.1016/j.metabol.2009.01.018
5. Wan, R., Camandola, S., & Mattson, M. P. (2003a). Intermittent fasting and dietary supplementation with 2-deoxy-D-glucose improve functional and metabolic cardiovascular risk factors in rats. *The FASEB Journal*. doi: 10.1096/fj.02-0996fje
6. Wan, R., Camandola, S., & Mattson, M. P. (2003b). Intermittent Food Deprivation Improves Cardiovascular and Neuroendocrine Responses to Stress in Rats. *The Journal of Nutrition, 133*(6), 1921-1929.

d. How many animals will be used and how did you determine that this will be a sufficient number?

i. Number of animals requested:

N=14, 7 per treatment group.

-

ii. How did you determine that this would be a sufficient number? (Choose at least one)

☒ Statistical power analysis (please describe)

Based on the relevant literature, 7-10 rats per group/time point is sufficient to detect a significant difference due to treatment. Using the standard deviation for leptin and ghrelin from a previous study, the power for n=7 and for n=10 is presented below.

Well designed studies typically generate a power of at least 0.8.

1. Ghrelin

	Fed	Fasted	Power for n=7	Power for n=10
Mean	14.9	19	100%	100%
SD	0.9	1.5		

1. Leptin

	Fed	Fasted	Power for n=7	Power for n=10
Mean	0.54	1.23	100%	100%
SD	0.1	0.4		

2. Ghrelin

		24h	Light	Dark	Peak
Free Fed	Mean1	1333.3	1549	1099.6	1857.7
	SD1	77.58	123.54	64.6	257.53
Restricted Feed	Mean2	1735.4	1247.7	2365.3	3649.8
	SD2	142.23	91.96	252.96	401.12
	Mean diff	-402.1	301.3	-1265.7	-1792.1
	Pooled SD	109.905	107.75	158.78	329.325
	Power for n=7	100%	100%	100%	100%
	Power for n=10	100%	100%	100%	100%

2. Leptin

		24h	Light	Dark	Peak
Free Fed	Mean1	3.5	2.6	4.6	6.2
	SD1	0.24	0.15	0.38	0.91
Restricted Feed	Mean2	5.3	6.6	3.7	7.8
	SD2	0.47	0.71	0.33	0.72
	Mean diff	-1.8	-4	0.9	-1.6
	Pooled SD	0.355	0.43	0.355	0.815
	Power for n=5	100%	100%	99.90%	97.70%
	Power for n=10	100%	100%	100%	99.70%

1. Bagnasco, M., P.S. Kalra, and S.P. Kalra, *Ghrelin and Leptin Pulse Discharge in Fed and Fasted Rats.* Endocrinology, 2002. **143**(2): p. 726-726.
2. Bodosi, B., et al., *Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation.* American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 2004. **287**(5): p. R1071-R1079.

☒ Similar published experiments (cite references)

(n=7):

Bagnasco, M., P.S. Kalra, and S.P. Kalra, *Ghrelin and Leptin Pulse Discharge in Fed and Fasted Rats.* Endocrinology, 2002. **143**(2): p. 726-726.

Bodosi, B., et al., *Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation.* American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 2004. **287**(5): p. R1071-R1079.

(n=8):

Wan, R., Camandola, S., & Mattson, M. P. (2003a). Intermittent fasting and dietary supplementation with 2-deoxy-D-glucose improve functional and metabolic cardiovascular risk factors in rats. *The FASEB Journal*. doi: 10.1096/fj.02-0996fje

Wan, R., Camandola, S., & Mattson, M. P. (2003b). Intermittent Food Deprivation Improves Cardiovascular and Neuroendocrine Responses to Stress in Rats. *The Journal of Nutrition, 133*(6), 1921-1929.

(n=10)

Castello, L., Maina, M., Testa, G., Cavallini, G., Biasi, F., Donati, A., . . . Chiarpotto, E. (2011). Alternate-day fasting reverses the age-associated hypertrophy phenotype in rat heart by influencing the ERK and PI3K signaling pathways. *Mechanisms of Ageing and Development, 132*(6–7), 305-314. doi: http://dx.doi.org/10.1016/j.mad.2011.06.006

☐ Special consideration for difficult to obtain, rare, or endangered species, explain for clarification

e. For 3-year renewals please describe what has been accomplished in the past 3 years and how this protocol differs from the previously approved protocol (for new protocols indicate N/A):

30. ASSURANCES (See Appendix B)

I have searched the following sources for alternatives to animal model.

Data Source	Searched From (Yr)	Searched To (Yr)	Keywords used in the Search	Date Search Performed
Pubmed	2000	2015	Alternate day fasting, intermittent fasting, leptin, ghrelin, body composition, body fat	7/20/2015
Medline via Web of Knowledge	2000	2015	Alternate day fasting, intermittent fasting, leptin, ghrelin, weight loss, body composition	7/20/2015
Biomed Central	2000	2015	Alternate day fasting, intermittent fasting, leptin, ghrelin, weight loss	7/20/2015

Alternatives refer to methods or approaches which result in refinement of procedures which lessen pain and/or distress; reduction in numbers of animals required; or replacement of animals with non-whole-animal systems or replacement of one animal species with another, particularly if the substituted species is non-mammalian or invertebrate.

I have determined that the following alternatives are available. Described briefly if applicable:

The dynamic changes in leptin and ghrelin levels due to alternate day fasting is best studied in an intact, whole-animal system. Replacement of animals with humans is not practical because of the inability to control diet. Dietary compliance and physical acitivity can drastically influence the levels of leptin and ghrelin, which is difficult to control in humans. Even with attempts to educate subjects on diet and physical activity parameters, it is still possible that subjects will erroneously record food intake or consume more during fasting days, which will affect the results.

Investigator Certifications

By submitting and digitally signing this package in IRBNet, you agree to the following certifications:

I understand that 'unnecessarily duplicative' research involving laboratory animals is not permitted by the federal animal welfare law and hereby provide assurance that the research proposed herein does not, to the best of my knowledge, unnecessarily duplicate research already reported in the public literature.

I certify that the use of all animals involved in this project will be carried out according to the provisions of the Animal Welfare Act, PHS Policy, the principals of the 'NIH Guide for the Care and Use of Laboratory Animals', and the UNLV Policy Governing The Use of Animals in Research, Teaching and Testing. I agree to notify the UNLV Institutional Animal Care and Use Committee (IACUC) of any substantive changes in the research use of the animals, including the number of animals, species used, or procedures performed.

I understand that the University of Nevada, Las Vegas and its representatives on the IACUC have the authority to suspend any part of my research, should I not be in compliance at any time with USDA, PHS/NIH, or UNLV regulations for animal care and use.

I certify that all personnel having direct animal contact, including the investigator, have or will have, prior to participating in this protocol, been trained in humane and scientifically acceptable procedures in animal handling, administration of anesthetics, analgesics, and euthanasia to be used in this project or are under the direct (in-lab) supervision of a trained individual; and that employees will be allowed adequate time to attend training sessions.

I assure that all named individuals on this project have read and understood the procedures outlined in this protocol as approved by the IACUC.

I certify that I will obtain approval from the IACUC before initiating any significant changes in this study.

I certify that I will notify the IACUC regarding any unexpected study results that impact the animals. Any unanticipated pain or distress, morbidity or mortality will be reported to the attending veterinarian and the IACUC.

I certify that the information provided in this application is accurate to the best of my knowledge. I also understand that should I use the project described in this application as a basis for a proposal for funding (either intramural or extramural), it is my responsibility to ensure that the description of animal use in such funding proposal is identical in principle to that contained in this protocol.

I understand that the Department of Laboratory Animal Care Services is responsible for administering and assigning animal housing space within the central animal facilities. The Manager, LACF will make space assignments for the efficient utilization of space, which may result in investigators sharing animal housing space. I further realize that I must notify the Manager, LACF of animal housing needs in order to insure the availability of suitable space before animals are procured.

APPENDIX II:

RAW DATA – BODY WEIGHT

Subject	Pre	Week 1	Week 2	Week 3	Week 4
1	169	227	273	318	343
2	179	232	260	343	378
3	173	222	257	288	312
4	183	244	294	337	373
5	181	236	291	326	362
6	177	233	293	335	372
7	174	225	278	321	346
Mean	176.57	231.29	278.00	324.00	355.14
SD	4.89	7.43	15.49	18.24	23.33
SE	1.85	2.81	5.86	6.89	8.82
8	187	200	258	265	319
9	171	188	236	238	288
10	169	175	220	223	259
11	166	188	239	237	283
12	181	187	239	245	305
13	167	183	236	246	293
14	174	179	227	233	285
Mean	173.57	185.71	236.43	241.00	290.29
SD	7.79	7.99	11.82	13.10	18.77
SE	2.94	3.02	4.47	4.95	7.09

RAW DATA – FOOD INTAKE

	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	9.6	18	20.3	19.2	21.5	19.8	17.1	23.3	22.6
2	13.1	17.1	18	21.5	19.5	22.1	19.2	23	22
3	16.2	17.9	15.2	19.7	19.2	18.9	19.2	17.2	18.6
4	19	18.8	20.1	21.2	19.4	23.9	24.6	20.6	24.1
5	18.7	16.7	20.9	21.4	20.3	20.6	23.3	22.6	23.4
6	16.5	18.1	18.2	19.4	18.3	21	19.6	22.8	21.1
7	13.5	17.3	22.3	21.8	20	20.4	39.3	20.2	24.1
8	4	22.9	4.1	27.2	4	25.7	4	27.7	4.3
9	4.1	20.2	4.3	22.1	4	23.7	4.1	26.2	4
10	4.3	20.5	4.3	25.2	4.2	24.3	4.1	28.6	4.3
11	3.9	21.6	4	27	4.1	27.6	4.1	27.6	3.9
12	4.1	24.3	4	28.8	4	26.8	4.1	30.4	4.1
13	4	20.3	4.2	24	4.3	23.6	4.2	24.6	4.2
14	4.1	19.4	4	21.3	4	22.2	3.9	23.6	3.9

	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20
1	18.1	21	23.9	22.8	22.5	23.8	19.3	23.6	22.4	25.5
2	22.2	22.4	24.6	25.2	22.3	23.6	25.4	25	24.8	26
3	18.6	21.2	16.8	16.7	18.2	22.2	19.7	19.6	19.9	21.2
4	25.3	21.7	26.3	23.9	22.1	25.7	25.8	23	28.3	27.8
5	30.1	22.5	22.1	22.9	24.4	24.7	23.2	22.6	22.6	27.3
6	21.2	22.6	25	22.8	25.8	25.1	26.7	21	23.1	25.7
7	21.9	20	23.5	21.1	23.7	24.1	22.6	22.2	24.2	23.7
8	27.5	5.1	27.9	5.3	28.6	5.4	32	6.1	34.7	6.1
9	24	5.2	26.6	5.3	27.7	5.3	31.5	6.2	32.3	5.9
10	25.5	5	22.5	5.3	28	5.6	29.3	6.1	27.6	6.1
11	29.7	5.2	29.7	5.2	29.4	5.9	31.3	5.9	34	6
12	29.7	5	30	5.2	29.7	5.9	31.3	6.1	32.1	5.9
13	25.5	5	25.2	5.5	26.7	5.3	27.7	6.2	28.7	6
14	23.8	4.9	27.1	5.4	27.4	5.6	29.9	6.1	29.6	5.9

	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28	Day 29	Day 30
1	24.3	26.8	25.9	21.8	25.2	22.4	26.4	28.7	21.7	18
2	25.7	26.9	22.1	24.7	25.4	25.4	26.7	26.9	24.4	15.5
3	19.5	18	21	19.9	20.7	19.2	19.9	23.3	16.4	19.2
4	28.6	23.4	31.4	23.3	28.8	28	27.3	31.2	17.9	18
5	24.2	24.2	27	26.2	28.8	23.1	26.4	28	19.1	17.6
6	27.7	25.8	30.8	26.6	22.2	24.1	25.9	26.5	25.3	21.9
7	25.3	26.4	23.9	22.7	25.6	24.6	21.8	27.3	21.5	19.9
8	33.5	6.1	33.9	5.9	29.5	6.2	33	6.2	32.1	5.9
9	30.6	6.1	33.2	6	29.3	6.2	28.4	6.3	30.4	6.2
10	27.2	5.9	28.6	5.8	26.3	6.2	28.4	5.9	26.8	6.2
11	31.7	6.1	32.7	5.9	29.2	6	28.2	6	30	6.2
12	33.8	6.2	36.9	6.3	34.2	6.1	31.8	6	32.3	6
13	29.6	6.2	32.6	6.1	28.5	6.1	29.7	6	30.8	6.1
14	28.1	6	30.5	6	29.6	6	28.9	6.1	29.6	6.3

RAW DATA – BLOOD GLUCOSE

Subject	Day 2	Day 30
1	95	88
2	78	107
3	87	81
4	92	88
5	100	82
6	88	84
7	85	82
Mean	89	87
SD	7.16	9.09
SE	2.71	3.44
8	86	50
9	86	58
10	90	79
11	77	70
12	91	59
13	89	52
14	89	54
Mean	87	60
SD	4.74	10.53
SE	1.79	3.98

RAW DATA – BODY FAT

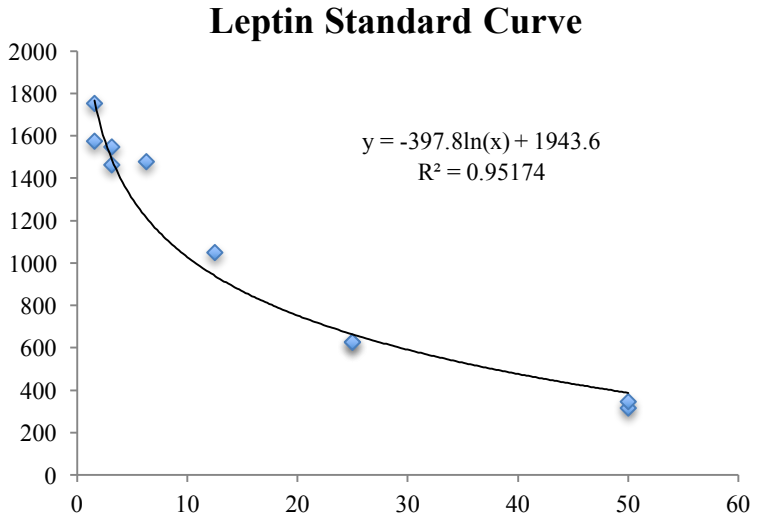
Subject	DEXA Body Fat %	Trunk % fat	Fat Pad Wt (g)
1	14.9	13.7	3.68
2	13.2	11.3	2.99
3	14	11.8	2.98
4	15.3	15.6	3.89
5	19.6	18.6	3.75
6	16.7	15.3	3.88
7	15.4	13.3	3.83
Mean	15.6	14.23	3.57
SD	2.088	2.508	0.407
SE	0.79	0.95	0.15
8	15.7	13.3	2.77
9	18.5	17.4	2.47
10	24.3	21.6	2.92
11	24.8	23.3	3.28
12	17.7	15.5	2.5
13	19.6	17.7	2.85
14	18.6	17	2.51
Mean	19.9	17.97	2.76
SD	3.406	3.436	0.294
SE	1.29	1.30	0.11

RAW DATA – BONE MINERAL DENSITY

Subject	BMD (g/cm^2)
1	0.168
2	0.157
3	0.137
4	0.166
5	0.14
6	0.153
7	0.134
Mean	0.151
SD	0.014
SE	0.005
8	0.138
9	0.135
10	0.135
11	0.134
12	0.139
13	0.133
14	0.124
Mean	0.134
SD	0.005
SE	0.002

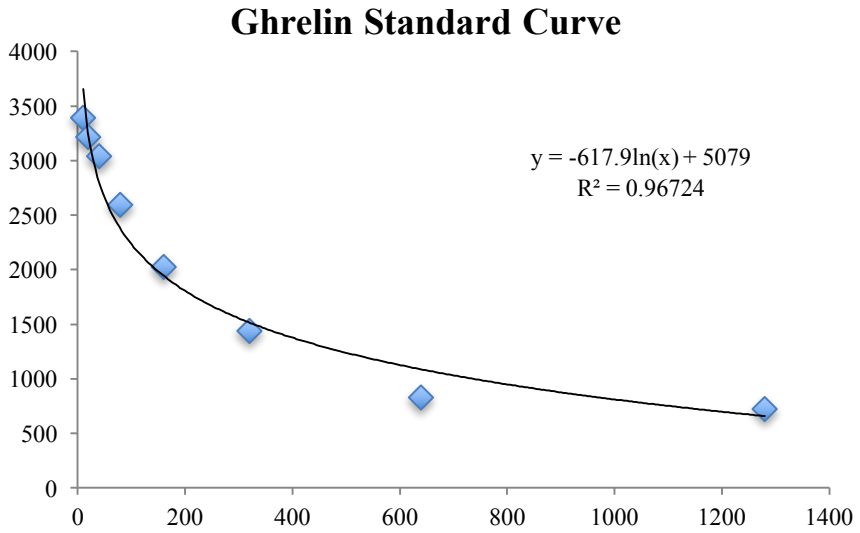
RAW DATA – LEPTIN

Subject	Day 1		Day 2		Day 3		Day 4	
	Average Count	ng/ml	Average Count	ng/ml	Average Count	ng/ml	Average Count	ng/ml
1	1160	7.17	1211	6.31	620.75	27.81	1137	7.60
2	720	21.67	1292.8	5.13	713.35	22.04	1208	6.35
3	1292	5.14	1115.5	8.02	692.2	23.24	1083	8.70
4	602.4	29.12	1139	7.56	866.7	14.99	1146.5	7.42
5	1276.2	5.35	1149	7.37	736	20.82	1057	9.29
6	658.4	25.30	1079	8.79	715.5	21.92	1055.5	9.32
7	795.9	17.91	1047.5	9.51	950	12.15	1098	8.38
Mean		15.95		7.53		20.42		8.15
SD		10.03		1.47		5.25		1.09
8	992	10.94	1147	7.41	972.5	11.49	995.1	10.85
9	1027.2	10.01	1293	5.13	828	16.52	1125.25	7.82
10	880.5	14.48	1214	6.26	610	28.57	1167	7.04
11	597	29.52	1176.5	6.88	617.9	28.01	1026	10.04
12	784.7	18.42	1220	6.17	742.25	20.49	1245	5.79
13	827.4	16.54	1095.5	8.43	733.75	20.93	1165.6	7.07
14	721	21.62	1161	7.15	633	26.97	963	11.76
Mean		17.36		6.78		21.85		8.63
SD		6.73		1.05		6.43		2.25



RAW DATA – GHRELIN

Subject	Day 1		Day 2		Day 3		Day 4	
	Average Count	pg/ml	Average Count	pg/ml	Average Count	pg/ml	Average Count	pg/ml
1	799	1019	992	746	1031	700	632	1336
2	800	1017	559	1504	806	1008	682	1233
3	748	1107	706	1186	828	973	786	1041
4	1233	505	731	1139	1027	705	791	1033
5	793	1029	972	770	1072	655	780	1051
6	734	1132	772	1065	632	1335	814	995
7	274	2385	401	1941	762	1082	489	1683
Mean		1170.70		1193.02		922.70		1196.07
SD		576.06		418.96		249.60		248.38
8	811	1000	741	1119	903	861	407	1923
9	933	820	714	1169	1141	586	467	1744
10	681	1234	505	1640	663	1270	607	1392
11	573	1469	532	1570	985	754	515	1615
12	907	855	446	1804	1093	633	798	1022
13	796	1025	830	970	978	763	641	1316
14	699	1199	895	872	742	1117	641	1316
Mean		1085.91		1306.53		854.81		1475.34
SD		229.60		361.47		251.91		304.48



APPENDIX III

STATISTICAL ANALYSIS

Body Weight – ANOVA

		Type III Sum of Squares	df	Mean Square	F	Sig.
Weight	Huynh-Feldt	192206.057	2.303	83448.275	638.084	.000
Weight * Diet	Huynh-Feldt	12532.057	2.303	5440.924	41.604	.000
Error(Weight)	Huynh-Feldt	3614.686	27.640	130.779		

Body Weight – Simple Main Effects

		Levene's Test		t-test for Equality of Means			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
WtPre	Equal variances assumed	1.393	.261	.863	12	.405	3.000
WtWeek1	Equal variances assumed	.001	.974	11.046	12	.000	45.571
WtWeek2	Equal variances assumed	1.342	.269	5.645	12	.000	41.571
WtWeek3	Equal variances assumed	.396	.541	9.778	12	.000	83.000
WtWeek4	Equal variances assumed	.641	.439	5.731	12	.000	64.857

Diet	Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Control	Weight	Huynh-Feldt	142884.000	2.272	62899.344	320.511	.000
	Error(Weight)	Huynh-Feldt	2674.800	13.630	196.247		
ADF	Weight	Huynh-Feldt	61854.114	2.410	25661.751	394.862	.000
	Error(Weight)	Huynh-Feldt	939.886	14.462	64.989		

Average Daily Food Intake – ANOVA

		Type III Sum of Squares	df	Mean Square	F	Sig.
Total Intake	Sphericity Assumed	159.335	2	79.667	127.865	.000
Total Intake * Diet	Sphericity Assumed	2.650	2	1.325	2.127	.141
Error(Total Intake)	Sphericity Assumed	14.953	24	.623		

Average Daily Food Intake – Pairwise Comparison

(I) Total Intake	(J) Total Intake	Mean Difference (I-J)	Std. Error	Sig.[b]
1	2	-3.511	.315	.000
	3	-4.553	.364	.000
2	3	-1.041	.188	.000

Average Fed Day Food Intake – ANOVA

		Type III Sum of Squares	df	Mean Square	F	Sig.
Fed Intake	Sphericity Assumed	185.710	2	92.855	87.890	.000
Fed Intake * Diet	Sphericity Assumed	7.361	2	3.681	3.484	.047
Error(Fed Intake)	Sphericity Assumed	25.356	24	1.056		

Average Fed Day Food Intake – Simple Main Effects

		Levene's Test		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
FedDay10	Equal variances assumed	1.135	.308	-4.745	12	.000	-4.51429	.95146
FedDay20	Equal variances assumed	.104	.752	-5.682	12	.000	-5.59429	.98452
FedDay30	Equal variances assumed	.062	.807	-6.019	12	.000	-6.56429	1.09064

Diet	Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Control	Fed Intake	Sphericity Assumed	60.444	2	30.222	31.700	.000
	Error(Fed Intake)	Sphericity Assumed	11.440	12	.953		
ADF	Fed Intake	Sphericity Assumed	132.627	2	66.314	57.186	.000
	Error(Fed Intake)	Sphericity Assumed	13.915	12	1.160		

Body Fat – Independent t-test

	Levene's Test		t-test for Equality of Means				
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Body Fat Equal variances assumed	1.952	.188	-2.848	12	.015	-4.30000	1.50979

Percent Trunk Fat – Independent t-test

	Control	***ADF***
Mean	14.22857143	17.97142857
Variance	6.292380952	11.80571429
Observations	7	7
Pooled Variance	9.049047619	
Hypothesized Mean Difference	0	
df	12	
t Stat	-2.327747327	
P(T<=t) two-tail	0.038226329	
t Critical two-tail	2.17881283	

Fat Pad Weight – Indpenedent t-test

	Control	***ADF***
Mean	3.571428571	2.757142857
Variance	0.165847619	0.086390476
Observations	7	7
Pooled Variance	0.126119048	
Hypothesized Mean Difference	0	
df	12	
t Stat	4.289636545	
P(T<=t) two-tail	0.001050957	
t Critical two-tail	2.17881283	

Bone Mineral Density – Independent t-test

	Control	*ADF*
Mean	0.150714286	0.134
Variance	0.000193238	0.000024
Observations	7	7
Pooled Variance	0.000108619	
Hypothesized Mean Difference	0	
df	12	
t Stat	3.000328785	
P(T<=t) two-tail	0.011059936	
t Critical two-tail	2.17881283	

Blood Glucose – t-tests

	Control (Post)	*ADF (Post)*
Mean	87.42857143	60.28571429
Variance	82.61904762	110.9047619
Observations	7	7
Pooled Variance	96.76190476	
Hypothesized Mean Difference	0	
df	12	
t Stat	5.162230337	
P(T<=t) two-tail	0.000236163	
t Critical two-tail	2.17881283	

	ADF (Pre)	*ADF (Post)*
Mean	86.85714286	60.28571429
Variance	22.47619048	110.9047619
Observations	7	7
Pearson Correlation	-0.209352803	
Hypothesized Mean Difference	0	
df	6	
t Stat	5.659799761	
P(T<=t) two-tail	0.001307211	
t Critical two-tail	2.446911851	

Leptin – ANOVA

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Leptin	Huynh-Feldt	1885.093	2.230	845.228	22.713	.000
Leptin * Diet	Huynh-Feldt	11.134	2.230	4.992	.134	.895
Error(Leptin)	Huynh-Feldt	995.945	26.763	37.213		

Leptin – Pairwise Comparison

(I) Leptin	(J) Leptin	Mean Difference (I-J)	Std. Error	Sig.[b]
1	2	9.505	2.256	.007
	3	-4.483	2.730	.759
	4	8.269	2.322	.024
2	3	-13.988	1.698	.000
	4	-1.236	.457	.115
3	4	12.751	1.640	.000

Ghrelin – ANOVA

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Ghrelin	Sphericity Assumed	1584223.768	3	528074.589	6.448	.001
Ghrelin * Diet	Sphericity Assumed	308700.196	3	102900.065	1.256	.304
Error(Ghrelin)	Sphericity Assumed	2948509.286	36	81903.036		

Ghrelin – Pairwise Comparison

(I) Ghrelin	(J) Ghrelin	Mean Difference (I-J)	Std. Error	Sig.[b]
1	2	-121.357	108.204	1.000
	3	239.571	107.610	.275
	4	-207.429	102.193	.391
2	3	360.929	110.485	.040
	4	-86.071	118.845	1.000
3	4	-447.000	100.700	.005

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CURRICULUM VITAE

Debra Kirsty Mortel Tacad, RDN, LD

9730 Pine Thickett Ave.
Las Vegas, NV 89147
(626) 272-2047
Debra.Tacad@unlv.edu

EDUCATION

2014-Present **M.S. Kinesiology:** Exercise Physiology
University of Nevada, Las Vegas, Las Vegas, NV

Oct. 2014 **Certified Registered Dietitian/Nutritionist**
Commission on Dietetic Registration, Chicago, IL

2009-2013 **B.S. Nutrition Sciences:** Dietetics
University of Nevada, Las Vegas, Las Vegas, NV

2007-2008 **Diploma Pâtisserie and Baking**
Le Cordon Bleu College of Culinary Arts, Los Angeles, CA

PROFESSIONAL EXPERIENCE

Aug. 2014 - Present – Graduate Assistant: *University of Nevada, Las Vegas*

Coordinator and Instructor of UNLV Nutrition Center:

- Develop, edit, and teach nutrition presentations regarding:
  - Weight Management
  - Cardiovascular Health
  - Diabetes Management
  - Sports Nutrition
  - Healthy Eating on a Budget
  - Nutrition Workshops
- Manage scheduling of events and classes for the Nutrition Center.
- Coordinate and present cooking demonstrations in conjunction with the Children's Heart Center of Nevada.
- Manage various undergraduate independent study projects.
- Coordinate grant-funded grocery store tours
  - Train instructors (dietetic interns and undergraduates).
  - Manage publicity – newspaper advertisements and flyers.
  - Manage scheduling of tours at 12 different locations.
- Provide nutrition education presentations to various UNLV sports teams and physical education classes, and other community affiliations.
- Manage and organize demographic sheets for grant maintenance.
- Provide one-on-one nutrition consultations for the Las Vegas community members.

- Contribute nutrition information as a Dietitian Consultant for the Las Vegas Review Journal

Oct. 2015 – Dec. 2015 – Part-time Instructor *University of Nevada, Las Vegas*

Special Topics in Nutrition – Healthy Cooking; NUTR 490

- Develop structured curriculum and recipes for a 6-week cooking demonstration class.

Jan. 2014 - Aug.2014 – Dietetic Intern *University of Nevada, Las Vegas*

- Learned the skills necessary to earn a position as a dietitian including nutrition assessment, patient education, food service management, and community nutrition assessment.

Aug. 2012 - Apr.2014 – Student Representative *Dairy Council of Utah/Nevada*

- Provided nutrition information to student athletes, coaches, and parents during Nevada Interscholastic Activities Association (NIAA) sporting events and community health fairs.

Aug. 2008 - Dec. 2013 – Assistant Pastry Chef *The Gourmet Cake Factory*

- Created multiple pastries and cakes for casinos and wedding chapels.
- Assisted with the creation and testing new recipes.
- Coordinated daily production and assisted with specialty cake production.
- Assisted in training new employees and culinary interns.
- Managed photos and content of company website.
- Created daily invoices for wholesale and retail clients.
- Created brochures and flyers for publicity.

GRANTS, SCHOLARSHIPS AND AWARDS

2016 Graduate and Professional Student Association Travel Grant; University of Nevada, Las Vegas funding to attend the John Milner Nutrition and Cancer Research Practicum at the National Cancer Institute ($300 funded)

2016 UNLV Exercise Physiology Travel Grant; University of Nevada, Las Vegas: funding to attend the John Milner Nutrition and Cancer Research Practicum at the Nation Cancer Institute ($300 funded)

2015 UNLV Exercise Physiology Travel Grant; University of Nevada, Las Vegas: Travel funding to present research in Southwest American College of Sports Medicine conference in Costa Mesa, California ($300 funded)

2015 Graduate and Professional Student Association Award; University of

Nevada, Las Vegas: Effect of Alternate Day Fasting on Leptin and Ghrelin in Rats ($825, funded)

2015 Certificate of Participation: Festival of Excellence; Southern Utah University

MEMBERSHIPS

Academy of Nutrition and Dietetics
- Sports, Cardiovascular, and Wellness Nutrition (SCAN) DPG
- Oncology Nutrition (ON) DPG
- Food and Culinary Professionals (FCP) DPG

Southern Nevada Dietetic Association
American College of Sports Medicine – Southwest Chapter

CERTIFICATIONS/TRAINING

2015 Laboratory Animal Training Association
- Humane Care and Use of Laboratory Animals (Base Module)
- Humane Care and Use of the Laboratory Rodent (Species Module)
- Aseptic Surgery and Perioperative Care of Rodents
- Occupational Health and Safety

2014 Nevada Department of Health and Human Services: Licensed Dietitian
2014 Collaborative Institutional Training Initiative:
- Biosafety training
- Bloodborne Pathogens training
- Chemical Hygiene training
- Hazard Communications training
- HIPAA training
- Personal Protective Equipment training
- Radiation Safety training
- Unsealed Sources training

2013 American Heart Association: CPR and AED certified

PUBLICATIONS

Montes, J., Stone, T.M., Manning, J.W., McCune, D., **Tacad, D.K**., Young, J.C., DeBeliso, M., Navalta, J.W. Using Hexoskin Weatable Technology to Obtain Body Metrics in a Trail Hiking Setting. *International Journal of Exercise Science.* 2015

ABSTRACTS & SCIENTIFIC PRESENTATIONS

Tacad, D.K., Young, J.C., Kruskall, L.J., Navalta, J.W., Tandy, R.D., Hickman, R., Hunger Games: The Effect of Alternate Day Fasting on Food Intake, Body Weight, and Leptin and Ghrelin in Rats. Annual Graduate & Professional Research Forum, Las Vegas, NV, 2016.

Koschel, T.L., Manning, J.W., **Tacad, D.K**., Montes, J., Tanner, E., McCune, D., Tovar, A., Taylor, J., Young, J.C., DeBeliso, M., Navalta, J.W. Moderate Altitude Acclimation has no

Effect on Respiratory Exchange Ratio, or Percent of CHO and Fat Utilization During a 1-Mile Trail Run. Annual Meeting of the Southwest American College of Sports Medicine, Costa Mesa, CA, 2015.

Navalta, J.W., Manning J.W., **Tacad, D.K**., Montes, J., Tanner, E., McCune, D., Koschel, T.L., Tovar, A., Taylor, J., Young, J.C., DeBeliso, M. Body Mass Index has no Effect on the Post Exercise Hypotension Response Following a Trail Run. Annual Meeting of the Southwest American College of Sports Medicine, Costa Mesa, CA, 2015.

Tanner, E., Manning, J.W., Taylor, J., Montes, J., McCune, D., Koschel, T.L., **Tacad, D.K**., Tovar, A., Young, J.C., DeBeliso, M., Navalta, J.W. Validation of Hexoskin Biometric Shirt to Cosmed K4b^2 Metabolic Unit in Adults During Trail Running. Annual Meeting of the Southwest American College of Sports Medicine, Costa Mesa, CA, 2015.

Tacad, D.K., Manning, J.W., Montes, J., Tanner, E., McCune, D., Koschel, T., Tovar, A., Taylor, J., Navalta, J.W., DeBeliso, M., Young, J.C. Post Exercise Hypotension Response in Non-Hypertensive Adults Following a Self-paced Trail Run. Annual Meeting of the Southwest American College of Sports Medicine, Costa Mesa, CA, 2015.

Navalta, J.W., Manning, J.W., McCune, D., Montes, J., Stone, T.M., LaComb, C.O., **Tacad, D.K**., Ciulei, M.A., Young, J.C., FACSM, DeBeliso, M., FACSM. Using Hexoskin Wearable Technology to Obtain Body Metrics in a Trail Hiking Setting. Annual Meeting of the National American College of Sports Medicine, San Diego, CA, 2015.

Tacad, D.K., Manning, J.W., LaComb, C.O., Stone, T.M., Montes, J., Navalta, J.W., DeBeliso, M., FACSM. Test-retest Reliability Of Physiological Responses To Self-paced Trail Hiking. Annual Meeting of the National American College of Sports Medicine, San Diego, CA, 2015.

REFERENCES

Laura Kruskall, PhD, RDN, LD, CSSD, FACSM, FAND
Associate Professor
Program Director, UNLV Nutrition Sciences
Director of Dietetic Internship
T: 702-895-4985
Email: laura.kruskall@unlv.edu

John Young, PhD, FACSM
Professor
UNLV Kinesiology and Nutrition Sciences
T: 702-895-1500
Email: jack.young@unlv.edu

James Navalta, PhD
Associate Professor
UNLV Kinesiology and Nutrition Sciences
T: 702-895-1500
Email: james.navalta@unlv.edu