THE METABOLIC, NEURAL, AND BEHAVIORAL OUTCOMES OF INTERMITTENT CALORIC DEPRIVATION IN DIET-INDUCED OBESE MICE

by

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ABSTRACT OF THE DISSERTATION

The metabolic, neural, and behavioral outcomes of intermittent caloric deprivation in diet-induced obese mice.

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Alternate day intermittent fasting (IMF) has recently gained traction as a popular dietary strategy to produce weight loss in obese individuals. In this dissertation, we investigate the metabolic, neural, and behavioral effects of IMF to further elucidate the mechanisms driving sustained weight loss. To produce diet-induced obesity, male mice were placed on an *ad libitum* 45% high fat diet (HFD) for 8 weeks. Animals were subsequently placed on one of four experimental diets for 4 weeks as follows: continuation of *ad libitum* HFD, IMF of HFD (IMF-HFD), switched to a 10% low fat diet (LFD), or IMF of LFD (IMF-LFD). IMF-HFD and IMF-LFD animals consistently lost body weight, 13-27% and 18-32% reduction respectively, compared to HFD animals. Oral glucose tolerance AUC was lower in IMF-HFD (~50%) while insulin tolerance AUC was reduced in IMF-HFD, LFD, and IMF-LFD (~22-42%). Norepinephrine content in the anterior portion of the medial hypothalamus was higher in IMF animals compared to HFD and LFD groups while only IMF-LFD was higher in the posterior portion. Furthermore, relative *Npy* gene expression was higher in IMF-HFD and

IMF-LFD compared to HFD and LFD mice. Neural activation of hypothalamic NPY neurons was measured following an acute glucoprivic challenge with 2-deoxy-D-glucose (2-DG). In the paraventricular nucleus, there was a significant increase in neural activation following 2-DG administration, but there were no differences between treatment groups given 2-DG. There were no differences in the arcuate nucleus nor were there differences in NPY/c-Fos double-labeling in either region. A subset of animals, which also including groups pair-fed to IMF animals (PF-HFD and PF-LFD) were used to evaluate feeding behavior during IMF and after 6 weeks of HFD re-feeding. At the end of the diet period, the first meal of the last feeding day was recorded. The first meal duration was longer in LFD and IMF-LFD mice compared to HFD. Additionally, IMF-HFD had a greater first meal size and faster rate of consumption than HFD animals. Average meal duration at the end of the diet period was longer in the low-fat diet groups compared to the HFD-groups. There were no meal pattern differences at the end of the HFD re-feeding period. In summary, these results suggest that IMF is an effective strategy for weight loss that produces specific alterations in hypothalamic signaling and that meal patterns are only transiently altered in diet-induced obese male mice.

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Gotthardt, J. D., & Bello, N. T. (2017). Meal pattern alterations associated with intermittent fasting for weight loss are normalized after high-fat diet re-feeding. Physiol Behav, 174, 49-56. doi:10.1016/j.physbeh.2017.02.046

Additional Publications

- **Gotthardt, J. D.**, & Bello, N. T. (2016). Can we win the war on obesity with pharmacotherapy? Expert Rev Clin Pharmacol, 1-9. doi:10.1080/17512433.2016.1232164
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- **Gotthardt, J. D.** and N. T. Bello (2014). "Efficacy and safety of lorcaserin–antiobesity effects with benefits." Journal of Symptoms and Signs 3(5).

CHAPTER 1:

Review of the literature

1. Review of the Literature

1.1 Importance of the problem

1.1.1. Overweight and obesity

Despite the existence of multiple evidenced-based therapies for the treatment of obesity, the prevalence of this disease continues to increase at an alarming rate. Obesity, as defined by the World Health Organization (WHO), is a disease in which excess adiposity accumulates to a degree that may be detrimental to both health and quality of life [1]. Specifically, a body max index (BMI) of $\geq$ 25 kg/m^2 is used to classify overweight and $\geq$ 30 kg/m^2 for obesity [1]. The prevalence of obesity has increased globally over the last several decades, currently affecting nearly 2 billion adults over the age of 18 [1]. The severity of this epidemic has garnered responses from prominent organizations such as the WHO, Centers for Disease Control and Prevention, US Department of Agriculture, the National Institutes of Health, and the Institute of Medicine.

Overweight and obesity are associated with a number of significant comorbidities, including diabetes, cardiovascular disease, cancer, dyslipidemia, osteoarthritis, and reproductive abnormalities [1, 2]. As such, there exists the potential for a marked reduction in quality of life and an increase in mortality risk for individuals with a BMI $\geq$ 25 kg/m^2. Developing strategies to target not only weight loss, but also weight loss maintenance, to prevent the development of comorbidities is of critical importance. Currently, there exist three main therapeutic avenues to treat obesity – pharmacotherapy, bariatric surgery, and behavioral modification [2].

1.1.2 Pharmacotherapy

The US Food and Drug Administration (FDA) criteria for approval require a mean efficacy, or placebo-subtracted, weight loss of 5% and at least 35% of drug-treated individuals maintaining a 5% weight loss from their initial weight compared to the placebo-treated group [3]. There are currently 6 medications approved by the FDA for the treatment of obesity. These include orlistat, phentermine/topiramate extended release, lorcaserin, naltrexone/bupropion extended release, and liraglutide [4]. Despite modest weight-reduction outcomes and moderate safety profiles, these drugs have largely failed to prevent or reduce obesity due to several significant obstacles. These limitations include but are not limited to side effects, prohibitive costs, and lack of patient education [4]. Pharmacotherapy is also prescribed in conjunction with, rather than as a replacement to, consuming a lower calorie diet and increasing physical activity. As such, finding ways to achieve and maintain adherence to a reduced calorie diet are of the utmost importance.

1.1.3 Bariatric surgery

If all other attempts at weight loss are ineffective, patients with a BMI of ≥40 kg/m^2 (also known as class III obese) or a BMI ≥35 kg/m^2 with one or more obesity-related comorbidities (i.e. non-insulin-dependent diabetes mellitus, hypertension, or sleep apnea) may choose to pursue bariatric surgical options [5]. The most common and clinically effective surgical options are Roux-en-Y gastric bypass (RYGB) and laparoscopic adjustable gastric banding (LAGB), in which up to 25% and 15% weight loss has been

documented 3-years after surgery, respectively [6-9]. Bariatric surgical options, however, are often accompanied by complications and exclude individuals with a BMI below 35 kg/m^2 that may need to achieve weight loss to improve comorbidities. Complications associated with RYGB and LAGB can range from nausea, vomiting, and dehydration [10] to increased mortality, alcohol dependency, and suicide [10-12]. Given the potentially severe unintended outcomes and invasive nature of bariatric surgery, alternative methods should be exhausted before considering this avenue of weight loss.

1.1.4 Lifestyle modification

Lifestyle modification, which is recommended prior to and concurrent with both pharmacotherapy and bariatric surgery, requires individuals to reduce overall calorie consumption (i.e. caloric restriction; CR). CR can be attained by reducing daily caloric intake by 15-40% [13]. Adhering to daily CR necessitates the use of diligent food records or diaries to achieve success, which can often be accompanied by feelings of restriction and frustration. Intermittent fasting (IMF) is an alternative approach to daily CR. With IMF, individuals alternate periods, varying from hours to days, of complete abstinence from food intake (fasting or caloric deprivation days) with periods of unrestricted food intake (feeding days) [14]. One benefit of IMF is that it does not require individuals to count calories. Thus, feeding days allow for a sense of freedom from any feelings of restriction [15]. Additionally, studies in human populations often allow for a small meal (approximately 15-20% of one's daily caloric need) on the fasting day, referred to as modified alternate day fasting, to reduce feelings of hunger and improve long term

adherence [13]. However, most pre-clinical trials of IMF have attempted to elucidate mechanisms driving the improvements in metabolic control observed in IMF using a complete fast (100% caloric deprivation) on the fasting day. For this reason, the works described in this thesis utilize a complete alternate day caloric deprivation model. Several studies by Wan et al. suggest that IMF improves metabolic parameters by imposing a mild, but beneficial, repeated stressor on animals [16, 17]. Wan and colleagues found that rats on an IMF chow diet gained less weight than *ad libitum* (AL) fed counterparts, had lower plasma levels of both insulin and glucose, and that IMF protected against damage induced by myocardial infarction [16]. One major limitation to previous rodent studies of IMF is that they were performed in non-obese or lean animals. Because obesity induces a distinct metabolic and neural phenotype [18-20], the effects of weight loss, or lack of weight gain, in lean animals does not accurately represent weight loss in obese animals. Thus, the goal of this thesis is to examine the metabolic, neural, and behavioral changes associated with IMF-induced weight loss in an obese mouse model.

1.2 Central control of energy homeostasis

1.2.1 Overview

Energy balance, or homeostasis, is achieved when the amount of energy expended by an individual (e.g. daily activity and exercise) is equal to the energy consumed through nutrition (e.g. kilocalories). When this balance is tipped, the individual will subsequently lose or gain body as a result of energy deficit or surplus, respectively. To achieve and maintain homeostasis, there are numerous neural pathways initiated in response to

situational cues, such as feeding, starvation, energy expenditure, excess energy intake, and ingestion of specific macronutrients [21]. Although there are other factors that influence feeding, such as environment, palatability of food, and reward, these will not be the focus of this doctoral thesis. Within the central nervous system (CNS), there are several regions directly involved in the regulation of energy homeostasis. Located in the hindbrain, the nucleus of the solitary tract (NTS) has long been implicated in its role in feeding behavior [21-26]. Further, the hypothalamus, which contains the arcuate nucleus (ARC), lateral hypothalamus (LH), ventromedial hypothalamus (VMH), and paraventricular nucleus (PVN), receives both projections from the NTS and direct endocrine signals from the periphery [22]. Coordinated excitatory and inhibitory responses from the NTS and hypothalamic nuclei work in concert to maintain balance in the face of homeostatic challenges.

1.2.2 Arcuate circuits

The ARC is located adjacent to either side of the third ventricle and extends down into the median eminence, a circumventricular organ with a fenestrated blood-brain barrier, see Figure 1 [27]. This unique position allows for circulating hormones and nutrients to directly bind to their receptors on ARC neurons and provide rapid feedback regarding energy status and nutrient availability. One group of neurons within the ARC co-expresses both neuropeptide Y (NPY) and agouti related protein (AGRP) and is considered orexigenic when activated [21, 28, 29]. These neurons will be referred to as “NPY neurons” here and throughout the dissertation. Conversely, a second neuron

population within the ARC counters the action of NPY neurons by promoting satiety and is considered anorexigenic [29, 30]. These neurons express pro-opiomelanocortin (POMC) and will be referred to as “POMC neurons”.

NPY was first discovered in 1982 by Tatemoto and colleagues [31]. It is 36 amino acids in length and a member of the pancreatic polypeptide family, which includes both pancreatic polypeptide (PP) and peptide YY (PYY) [32]. Though NPY is widely expressed throughout the brain and associated with a multitude of processes, NPY neurons within the hypothalamus have potent orexigenic effects when activated. Numerous studies have demonstrated a significant increase in feeding when exogenous NPY is centrally administered [33-35]. Moreover, when NPY signaling is disrupted by intracerebroventricular (i.c.v.) injections of NPY antibodies, non-fasted overnight food intake and cumulative 24-hour intake are significantly reduced compared to control animals [36]. Interestingly, mice lacking the NPY gene maintain normal body weight and food intake under fed conditions [37], but demonstrate an attenuated hyperphagic response to fasting [38]. There are six known G-protein coupled Y receptors (Y_1-Y_6) with varying affinities for NPY, PP, and PYY and elicit diverse functions [39]. The Y_1 and Y_5 receptors are thought to be largely responsible for NPY’s orexigenic effects and are located throughout the hypothalamus. In direct contrast to NPY, hypothalamic POMC release functions to decrease feeding. POMC, once released, undergoes a series of cleavages to produce α-melanocyte stimulating hormone (α-MSH). α-MSH binds to the melanocortin 3 and 4 receptors (MC3R and MC4R, respectively) in the PVN and LH to

reduce feeding [40, 41]. AGRP works to promote feeding through its antagonism of both MC3R and MC4R, thus inhibiting satiety signaling, see Figure 1.

1.2.3 The paraventricular nucleus

The PVN is also located adjacent to either side of the third ventricle and dorsal to the ARC, see Figure 1. MC4R is abundantly expressed in the PVN and is one of the ways in which energy balance is controlled in this location [40]. When an exogenous MC4R agonist (MTII) is administered through an intracerebroventricular (i.c.v.) injection for 14 days, food intake is immediately and transiently reduced to approximately half of control animals, but returns to control intake by day 11 [42, 43]. Conversely, long term i.c.v. administration of the MC4R antagonist SHU-9119 progressively increases average intake to almost double that of vehicle treated animals [42]. NPY neurons from the ARC project to the PVN to promote feeding behavior, in part, through antagonism of MC4R [44]. In addition to energy balance, the PVN also regulates stress and thyroid function [45, 46].Though predominantly known for their role in stress and thyroid regulation, the PVN also contains corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) containing neurons, which have also been implicated in feeding behavior [47, 48]. Indeed, central injection of exogenous CRH into the PVN of Sprague-Dawley rats decreases food intake up to 2 hours post-injection [49]. Furthermore, subcutaneous injection of TRH in rats results in acute, 24-hr reductions of food-intake that do not persist when administered daily for 5 days [50].

1.2.4 NPY during caloric restriction and IMF

Hypothalamic NPY expression is highly influenced by energy status. Arcuate NPY neurons are glucose sensitive and their activity reduced in response to elevated blood glucose levels. This is highlighted by the fact that intravenous infusion of glucose is sufficient to decrease NPY stimulated feeding [51]. Conversely, reductions in blood glucose increase NPY activity. Food deprivation of 48-96 hours will cause a dramatic and significant increase in NPY protein expression in both the PVN and ARC nuclei compared to satiated animals [52-54]. Upon refeeding, NPY levels will return to baseline or those of fed rats. Likewise, caloric restriction, without complete deprivation, is sufficient to increase hypothalamic NPY gene expression [55, 56]. Bi and colleagues demonstrated that when rats are calorically restricted (30% reduction from AL fed controls) either acutely (48-hr) or long term (14 days), ARC NPY and AgRP mRNA levels are significantly greater than controls while POMC levels are reduced [56]. There is minimal evidence available regarding the expression and regulation of NPY during IMF in obese individuals. However, studies performed in non-obese animals may provide some clues as to how NPY neurons respond to an IMF style of dieting. The first study to look at NPY during IMF was published by Kumar and Kaur in 2013 using non-obese, 3-4 month old, male and female Wistar rats [57]. Rats were placed on an alternate day style of IMF for 12 weeks in which alternating 24-hr periods of food deprivation and AL access to food. Both male and female rats on IMF showed significant increases in the intensity of arcuate NPY immunostaining and non-significant increases in arcuate NPY gene expression compared to controls [57]. These data, however, are confounded by the fact that IMF animals were sacrificed on a fasting

day and controls were not fasted prior to sacrifice. Thus, it is unclear if this upregulation of NPY production is a result of IMF or that the control was in a fed state. Another study by Chausse and colleagues, published in 2014, was performed in a similar manner using 8-week old, male Sprague-Dawley rats for 3 weeks [58]. In this study, qRT-PCR was performed on tissue from fed and fasted animals from both IMF and control groups. Interestingly, they found that NPY mRNA expression was significantly increased in IMF animals only in the fed state [58]. Likewise, AgRP expression was significantly higher in IMF animals in both fed and fasted states. These findings suggest that perhaps the repeated bouts of caloric deprivation experienced during IMF result in a latent downregulation, or a chronic upregulation, in NPY expression that remains even after refeeding occurs.

1.2.5 Role of norepinephrine in feeding behavior

The noradrenergic system, which includes seven norepinephrine (NE) containing nuclei (A1-A7), is responsible for both stimulating and inhibiting feeding behavior and is heavily involved in energy homeostasis. This system originates from the hindbrain and projects to many locations within the CNS [25, 59]. The A1 and A2 nuclei are thought to be the primary NE containing neurons that project to the ARC, PVN, and LH within the hypothalamus [23, 25, 60, 61]. Specifically, A2 neurons, which originate in the NTS, are responsible for the integration peripheral signals from vagal afferents with hunger and satiety signals from the hypothalamus [30, 62, 63]. There is evidence to suggest that NE signaling from the hindbrain is altered in obesity. DIO rats given an intraperitoneal (i.p.)

injection of the selective NE reuptake inhibitor, nisoxetine, produced a significantly greater 24-hr reduction in food intake than control animals [64]. Moreover, DIO rats given nisoxetine had significantly greater c-Fos positive cells, a marker of neural activation, in the ARC compared to non-DIO controls [64]. A maternal investigation of obesity found that offspring from DIO dams expressed significantly higher levels of the NE transporter in the PVN, ARC, and VMH compared to offspring from non-DIO dams [65]. More data are needed to clarify, not only the exact changes in NE signaling that occur in obesity, but also how weight loss may influence the noradrenergic system.

1.2.6 NE and NPY during glucoprivation

It has been known for nearly half a century that restricting glucose utilization (i.e. glucoprivation) stimulates a feeding response [66, 67]. Glucoprivation, which occurs during homeostatic challenges such as fasting, can be induced by i.p. or i.c.v. injection of the non-metabolizable glucose analog 2-deoxy-D-glucose (2DG). 2DG restricts glycolysis and depletes the cell of ATP at several points in the glycolytic pathway. First, 2DG utilizes the same intracellular glucose transporters as glucose thereby competitively inhibiting glucose uptake into the cell [68]. Once inside the cell, 2DG competitively binds to hexokinase and is converted to 2-deoxy-D-glucose-6-phosphate (2DG-6P), which cannot be metabolized by phosphoglucose isomerase but competitively inhibits its conversion of glucose-6-phosphate to fructose-6-phosphate [68], see Figure 2. As the cell can no longer adequately produce ATP through either glycolysis or oxidative phosphorylation and as ATP is required for the conversion of 2DG to 2DG-6P, ATP stores rapidly become depleted

and cell death can occur if administered repeatedly [68]. Acute 2DG administration, however, is sufficient to deprive peripheral tissues of glucose and induce hyperphagia. There is a substantial body of evidence to support the involvement of both NE and NPY in the hyperphagic response to glucoprivation [69-79]. Early studies demonstrated that central 2DG injection resulted in neural activation, via c-fos expression, within the PVN and ARC of the hypothalamus [71, 78]. More recently, work from the Ritter lab using anti-dopamine β-hydroxylase antibodies conjugated to saporin (DSAP) has demonstrated a causal relationship between NE signaling, NPY, and glucoprivic feeding.

Saporin is an immunotoxin that can be used to lesion targeted neuron populations when conjugated to a specific peptide or antibody [80]. Dopamine β-hydroxylase is the enzyme responsible for the conversion of dopamine to norepinephrine. Thus, DSAP selectively targets and destroys neurons that contain dopamine β-hydroxylase (i.e. norepinephrine and epinephrine containing neurons). When DSAP is administered directly to the PVN of rats, destroying noradrenergic terminals, glucoprivation induced by both 2DG and insulin fails to produce a hyperphagic response [81]. This finding holds true for rats given 2DG when DSAP is injected into the ARC [74]. Moreover, 2DG elicits increased NPY and AgRP gene expression in control rats but not in those administered DSAP to the ARC, suggesting that these neurons are necessary for the glucoprivic production of NPY and AgRP [74].

1.3 Peripheral signals that regulate energy homeostasis

1.3.1 Glucose regulation

Impaired glucose regulation is one of the hallmarks of metabolic syndrome and the co-morbidities associated with obesity. Impaired glucose regulation is typically characterized by elevated fasting plasma glucose (≥ 126 mg/dL) and insulin resistance in humans [2]. Endogenous insulin is produced in pancreatic β-cells and released postprandially to promote glucose uptake in peripheral tissues [82]. Achieving and maintaining normoglycemia is one of the major outcome goals of most obesity therapies [2]. As such, glucose regulation has been extensively studied in pre-clinical models of intermittent fasting. At present, there are currently conflicting data as to whether IMF improves or has negligible impacts on glucose control. In 2003, Mattson *et al*. demonstrated that 9-week old male C57BL/6 mice on an alternate day IMF schedule of chow for 20 weeks exhibit significantly reduced blood glucose and insulin concentrations following a 14-hour fast when compared to AL and pair-fed controls [83]. Likewise, Sprague-Dawley rats on an alternate day IMF of chow have lower blood glucose and insulin concentrations after an overnight fast than AL fed controls after both 3 and 6 months of IMF [16, 17, 57]. Conversely, this same group reported no differences in blood glucose or insulin following an overnight fast between IMF and AL male and female rats within each sex after 6 months of dietary intervention [84, 85]. Others have also shown no effects of IMF on blood glucose following an overnight fast [58, 86, 87].

One approach to delineate differences in glucose control is to perform a glucose tolerance test in which rodents are challenged with glucose and clearance is determined by recording blood values over the subsequent 2-3 hours. In a similar fashion, an insulin tolerance test to determine insulin responsiveness can also be performed. A tolerance

test provides insight as to the function of a metabolic system whereas a single sampling provides only a snapshot of a system at a single point in time. Such assays would be useful in clarifying the differences observed between studies of IMF thus far. Additionally, the current body of evidence regarding IMF and glucose homeostasis in pre-clinical models is in non-obese or lean animals. Because glucose homeostasis is often dysregulated in obesity, non-obese models do not accurately reflect the effects of intermittent fasting on weight loss. More work is needed to establish IMF-related outcomes on glucose regulation.

1.3.2 Insulin/Leptin signaling to the hypothalamus

In addition to its role in glucose uptake, insulin also serves as a powerful satiety and adiposity signal to the ARC of the hypothalamus. Both NPY and POMC neurons express the insulin receptor to either negatively or positively affect expression, respectively, and promote satiety [88]. When endogenous insulin production is disturbed through destruction of the pancreatic β-cells using streptozotocin (STC), rats become hyperphagic, less responsive to NPY, and increase NPY expression [88]. Thus, maintaining sensitivity to insulin is not only important for glucose uptake, but also for keeping satiety signaling intact. At present, it is unknown how insulin responsivity and receptor abundance are affected by IMF beyond single fasted plasma sample concentrations.

A second peptide of interest in the metabolic control of obesity is the adipokine leptin, which is released into the bloodstream in quantities directly proportional to fat mass to decrease food consumption. The leptin receptor is present throughout the

hypothalamus in the PVN and on both NPY and POMC neurons [88]. Deletion of the leptin gene (ob/ob mouse) or presence of a defective leptin receptor (db/db) results in a hyperphagic and obese phenotype in which only the former can be resolved through leptin administration [89]. Administration of exogenous leptin produces a decrease in body weight and food intake in non-obese animals, but fails to do so in obese once due to leptin receptor resistance [89]. Quantification of endogenous leptin, though not a measurement of response, can give information on adiposity and if exceptionally high, resistance. Six months of alternate day IMF of a chow diet in male, but not female, rats significantly reduces plasma insulin concentrations compared to AL fed controls [84]. Others have shown 3 months of IMF sufficient to significantly decrease plasma leptin levels in both sexes and that in females, leptin concentration is dependent on estrous phase [57]. Interestingly, IMF for 3 months in male Wistar rats also reduces leptin receptor expression in the hypothalamus compared to AL fed controls [87]. Chausse et al. have also attempted to elucidate leptin sensitivity following a 3-week IMF intervention in male rats [58]. Following a 12-hr fast, animals were given an i.p. injection of leptin (100 $\mu L/10^{-6}M$) or saline [58]. Not only were 4-hr cumulative food intakes significantly higher in IMF animals than AL ones given saline, but only IMF animals showed significantly lower total consumption as a result of leptin administration [58]. This finding suggests that IMF improves leptin sensitivity compared to AL animals.

1.3.3 Additional peripheral peptides

Similarly to insulin and leptin, the peripheral peptide glucagon-like peptide 1 (GLP-1) is an anorexigenic peptide. Preproglucagon is produced at several locations throughout the body, including the gut, pancreas, and NTS [90]. Preproglucagon is then cleaved into several products including glucagon, glicentin, oxyntomodulin, GLP-1, and GLP-2, depending on the location of the cleavage [28, 91]. The anorexigenic potencty of GLP-1 is demonstrated through the anti-obesity medication, liraglutide. Liraglutide shares approximately 97% homology with GLP-1, differing only at positions 26 (palmitoyl C-16 is bound to position glutamate) and 34 (lysine to arginine substitution) [92-94]. There is conflicting evidence regarding GLP-1 regulation in obesity and it is currently unknown if or how GLP-1 secretion is modified by IMF [95].

Of all the peripheral peptides, only ghrelin is considered orexigenic. Fasting increases serum concentrations of ghrelin and both central and peripheral administration produce increased food consumption and weight gain [96, 97]. Ghrelin is released by the gastrointestinal (GI) tract and binds to the growth-hormone secretagogue receptor (GHSR) [88, 96]. NPY neurons in the ARC express GHSR and i.c.v. administration of ghrelin results in increased NPY and AGRP gene expression [88, 98, 99]. IMF does not appear to influence fasted plasma levels of ghrelin in male rodents [58, 84], but significantly reduces ghrelin plasma levels in female rats after 6 months of an IMF diet [84]. Moreover, obesity may induce ghrelin resistance in ARC NPY neurons [100]. It is possible there would be a more robust difference in plasma ghrelin in IMF animals undergoing weight loss or that differences may be identified by looking at hypothalamic GHSR expression.

1.4 Meal patterns

1.4.1 Measuring and reporting meal patterns

Meal patterns are a biologically relevant, quantitative measure of metabolic health and satiety signaling [101]. These patterns include time to meal termination (i.e. meal duration), meal size (kilocalories consumed), meal frequency (number of meals in a given time period), and latency to eat (i.e. time between meals) [101]. Meal patterns can be influenced by a number of determinants, including central and peripheral signals, external/environmental stimuli, and physiological status. Centrally, NPY and NE exert a significant amount of control over feeding behavior. High carbohydrate meals have been shown to increase ARC NPY gene expression and NPY immunoreactivity in the ARC and PVN [102]. Conversely, exogenous NPY administration into the PVN increases carbohydrate consumption [103]. Moreover, exogenous NPY into the PVN of rats increases the size and duration of meals consumed but not frequency, indicating that NPY likely exerts its orexigenic effects by reducing the satiating effect of food [103]. Overexpression of NPY within the hypothalamus yields marginally different effects on feeding. Within the PVN, overexpression of NPY produces an increase in the total number of meals consumed but not meal size and within the LH, overexpression yields a greater meal size [104]. Likewise, hypothalamic NE administration increases consumption specifically through larger meal sizes and also meal size, duration, and rate of the first meal following injection [105].

Vagal afferent fibers to the hindbrain are critical for the integration of peripheral signals into the CNS. These fibers innervate the GI tract (i.e. esophagus, stomach, and

intestine) to relay ingestion information to the NTS [106]. The dorsal motor nucleus (DMV) subsequently conveys motility and satiety signals through vagal efferent fibers to the GI tract [106]. Peripheral peptides produced in the GI tract, such as cholecystokinin and PYY, exert an anorectic effect post-prandially, in part through the vagus nerve, to reduce meal size or time between meals [82, 107, 108]. Other peptides, such as leptin, can regulate long-term energy intake and reflect metabolic status such as level of adiposity [82, 101]. Leptin deficient mice, which are obese and hyperphagic, consume larger meals than their lean counterparts[109]. Obesity is associated with a dysregulation of peripheral and central signaling, such as leptin insensitivity or resistance, and increased overall intake attributed to larger meal sizes [108, 110, 111]. Subtle changes in meal patterns can contribute substantially to increases or decreases in overall caloric intake. Understanding the mechanism by meal patterns are influenced in obesity provides an avenue for developing more targeted therapies to improve weight loss and weight loss maintenance outcomes.

1.4.2 IMF and meal patterns

Though there is no comprehensive analysis of meal patterns during or after IMF, there are some concerns regarding and disordered eating behavior. Indeed, caloric deprivation and restriction are often utilized in models of binge eating disorder [112-115]. Several studies in rodents have demonstrated a propensity for hyperphagia at the termination of a fasting period [58, 116]. Animals intermittently fasted every 2-5 days, though not alternate day IMF, are able to consistently consume more on the 1-3 days

following a fast than AL animals without consuming significantly more cumulatively [116]. Similarly, rats on alternate day IMF for 3 weeks significantly increases 2-hour food intake following a fast compared to AL fed animals at the same time yet maintained lower cumulative intakes than AL animals [58]. Thus, despite overeating when food is available, animals on an IMF diet are not able to fully compensate for the deficit achieved. However, it is yet to be evaluated how this cycle of fasting and overeating will affect long term behavior following the termination of an IMF diet.

1.5 Summary of Objectives

This dissertation investigates the metabolic, central, and behavioral outcomes of intermittent fasting/caloric deprivation (IMF) in a diet-induced obese (DIO) mouse model. The objectives of the dissertation are as follows:

***Objective 1*: To determine how IMF alters metabolism, hypothalamic monoamine concentrations, and hypothalamic expression of genes related to metabolism.** DIO male mice were placed on *ad libitum* or IMF of a high or low fat diet. Glucose metabolism, respiratory exchange ratio, plasma hormones, and neural signaling were assessed. (Chapter 2)

Objective 2: To determine the effects of IMF on glucose-depending neural activation in the ARC and PVN of the hypothalamus. As with Objective 1, DIO male mice were placed on *ad libitum* or IMF of a high or low fat diet. Animals received an injection of the non-metabolizable glucose analog 2-deoxy-D-glucose or saline. Immunohistochemistry was used to determine neural activation. (Chapter 3)

<u>Objective 3</u>: To determine if feeding behavior is altered by IMF during a diet period and after high-fat diet refeeding in DIO male mice. Meal patterns were recorded and analyzed using the BioDAQ system. The first meal following a fast and meal size, meal duration, and meal frequency of a feeding day were assessed. (Chapter 4)

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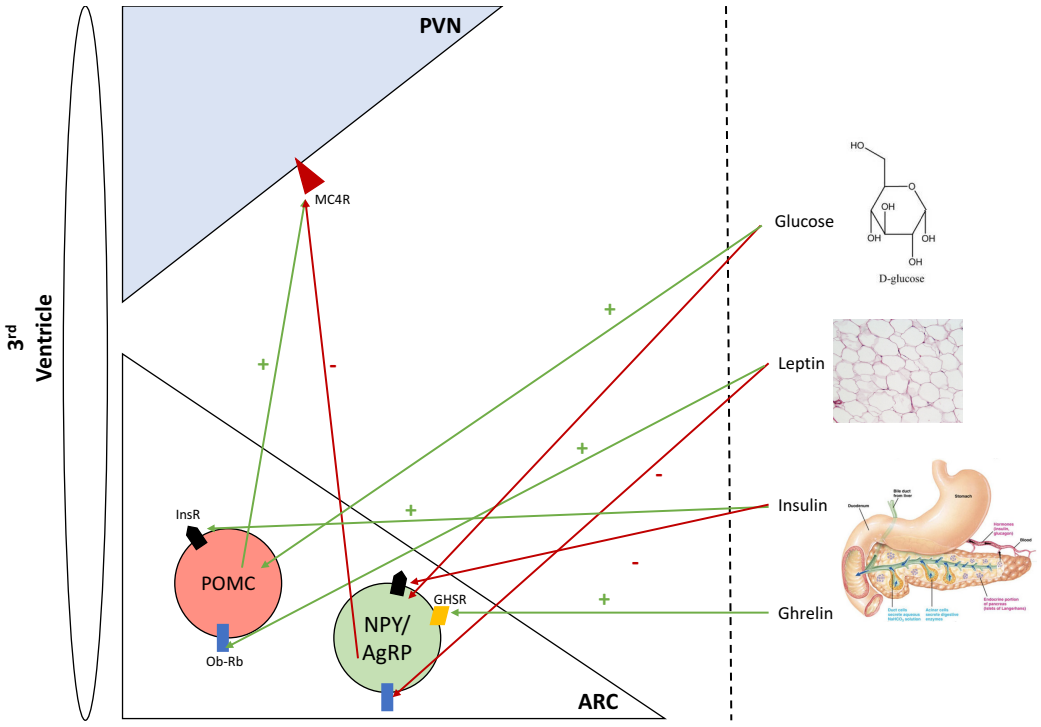
Figures

Figure 1. The arcuate (ARC) and paraventricular (PVN) nuclei of the hypothalamus are located adjacent to the third ventricle. In the ARC, there are neuropeptide Y (NPY)/agouti-related protein (AGRP) containing neurons, which are orexigenic, and pro-opiomelanocortin containing neurons (POMC), which are anorexigenic. The action of POMC at the melanocortin 4 receptor (MC4R) in the PVN is blocked by AGRP. Leptin, which originates from adipose tissue, binds to the leptin receptor (Ob-Rb) on both NPY and POMC neurons to reduce food intake proportional to adipose stores. Insulin from the pancreatic beta cells also reduces food intake by binding to the insulin receptor (InsR) in the ARC. POMC and NPY neurons are also glucose sensitive, decreasing food intake with increasing blood glucose levels. Conversely, ghrelin, which is produced in the stomach increases hunger pre-prandially by binding to the growth hormone secretagogue receptor (GHSR) on NPY neurons.

Figure 2. 2-deoxy-D-glucose (2DG) inhibits cellular glucose utilization and depletes the cell of ATP at several. Cellular glucose uptake is inhibited at the level of the glucose transporters (GLUTs) where 2DG competitively binds. Once within the cell, 2DG also competes for the glycolytic enzymes hexokinase (HK) and phosphoglucose isomerase (PGI). 2DG-6-P cannot be converted to fructose-6-P, ending that cycle of glycolysis, but accumulating in the cell. Further, 2DG blocks intracellular protein glycosylation as fructose-6-P is part of the glycosylation pathway. *This figure was originally published by D. Zhang et al. 2014* [68].

Figure 3. Peripheral signals (e.g. insulin, leptin, ghrelin, and glucose) and gastrointestinal peptides (e.g. cholecystokinin and ghrelin) relay information directly to the hypothalamus and through the vagus nerve, respectively. Vagal afferents are received at the nucleus of the solitary tract (NTS) and are transmitted throughout the hypothalamus to the arcuate nucleus (ARC), lateral hypothalamus (LH), and the paraventricular nucleus (PVN). Coordinated signals from the hypothalamus to the dorsal motor complex (DMV) are also processed and transmitted through vagal efferent fibers. *This figure was modified from Morton et al. 2006* [21].

Figure 1.


PVN
3rd Ventricle
MC4R
InsR
POMC
Ob-Rb
NPY/
AgRP
GHSR
ARC
Glucose
HO
O
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OH
OH
OH
D-glucose
Leptin
Insulin
Ghrelin
+
-

Figure 2.

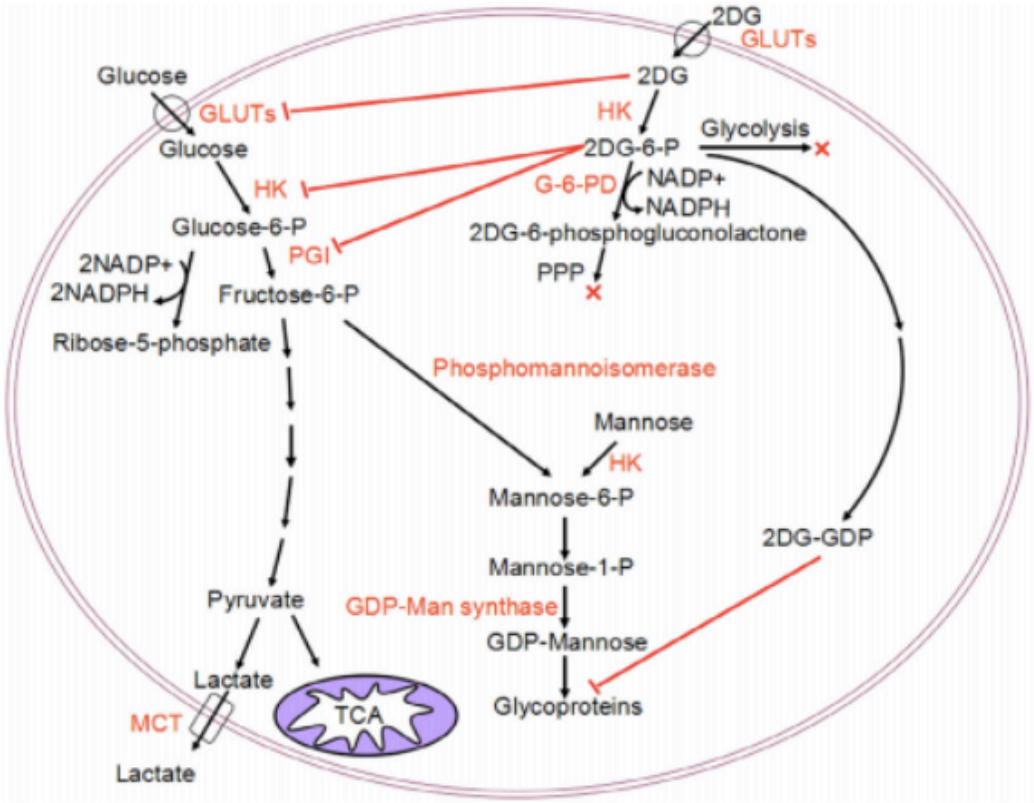
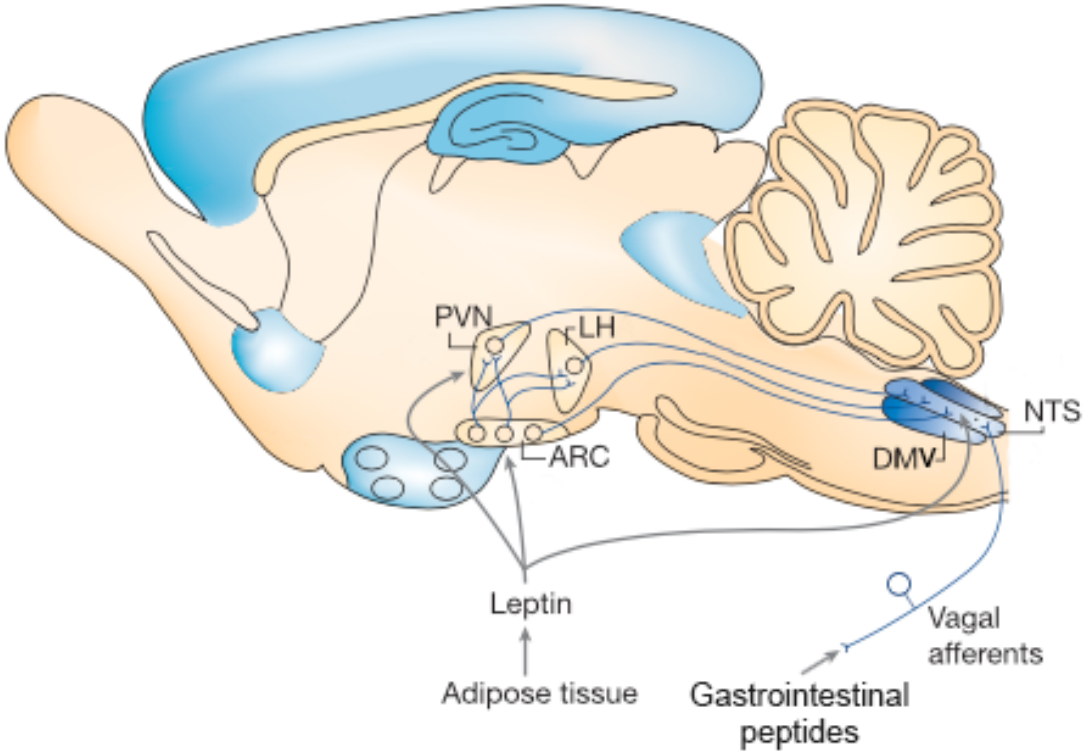

2DG
GLUTs
Glucose
2DG
GLUTs
HK
Glycolysis
Glucose
2DG-6-P
HK
G-6-PD
NADP+
NADPH
Glucose-6-P
2DG-6-phosphogluconolactone
PGI
2NADP+
2NADPH
PPP
Fructose-6-P
Ribose-5-phosphate
Phosphomannoisomerase
Mannose
HK
Mannose-6-P
2DG-GDP
Mannose-1-P
Pyruvate
GDP-Man synthase
GDP-Mannose
Lactate
TCA
MCT
Glycoproteins
Lactate

Figure 3.


PVN
LH
NTS
ARC
DMV
Leptin
Vagal
afferents
Adipose tissue
Gastrointestinal
peptides

CHAPTER 2:

Intermittent fasting promotes fat loss with lean mass retention, increased hypothalamic norepinephrine content, and increased neuropeptide Y gene expression in diet-induced obese male mice

2. Intermittent fasting promotes fat loss with lean mass retention, increased hypothalamic norepinephrine content, and increased neuropeptide Y gene expression in diet-induced obese male mice

2.1 Abstract

Clinical studies indicate alternate day, intermittent fasting (IMF) protocols result in meaningful weight loss in obese individuals. To further understand the mechanisms sustaining weight loss by IMF, we investigated the metabolic and neural alterations of IMF in obese mice. Male C57/BL6 mice were fed a high-fat diet (HFD; 45% fat) *ad libitum* for 8 weeks to promote an obese phenotype. Mice were divided into 4 groups and either maintained on *ad libitum* HFD (HFD), received alternate day access to HFD (IMF- HFD), switched to *ad libitum* low fat diet (LFD; 10% fat), or received IMF of LFD (IMF- LFD). After 4 weeks, IMF-HFD (~13%) and IMF-LFD (~18%) had significantly lower body weights than HFD. Body fat was also lower (~40-52%) in all diet interventions. Lean mass was increased in the IMF-LFD (~12-13%) compared with HFD and IMF-HFD groups. Oral glucose tolerance AUC was lower in the IMF-HFD (~50%), whereas insulin tolerance AUC was reduced in all diet interventions (~22-42%). HPLC measurements of hypothalamic tissue homogenates indicated higher (~55-60%) norepinephrine (NE) content in the anterior regions of the medial hypothalamus of IMF compared with *ad libitum* fed groups, whereas NE content was higher (~19-32%) in posterior regions in the IMF-LFD group only. Relative gene expression of *Npy* in the arcuate nucleus was increased (~65-75%) in IMF groups. Our novel findings indicate that intermittent fasting produces alterations in hypothalamic

NE and NPY, suggesting an involvement in the counter regulatory processes of short-term weight loss are associated with an IMF dietary strategy.

2.2 Introduction

Calorie restriction is the most widely prescribed and self-imposed strategy for treating excessive weight gain and obesity [1-3]. In the US, most common commercial programs for calorie reduction include reducing daily caloric intake by portion control, low calorie meals, and/or meal-replacement options [1]. Rather than reducing daily total caloric intake, intermittent fasting (IMF) has received attention as a possible approach for long-term weight loss [4]. Although varying in period of fasting (e.g., alternate day fasting or once/twice a week fasting days), IMF protocols have a similar advantage in that bouts of unrestricted eating occur following fasting periods [5, 6]. Several human studies have indicated that a short-term (i.e., 8-24 weeks) IMF protocol results in weight loss (i.e., 3-8%) in overweight or obese subjects [5-13]. Weight loss occurs over several weeks because, despite overeating on refeeding days, individuals do not fully compensate for the calorie-deficit realized on the fasting days [6]. One appealing feature of IMF protocols is that dieters do not have to count calories during the bouts of unrestricted eating [4]. However, one common obstacle to the long-term adherence to IMF is intense feelings of hunger during the fasting periods [14]. These subjective feelings of hunger can be mitigated by reducing the period of fasting or providing a small meal [10].

The influence of IMF on the hypothalamic control of energy homeostasis in obesity provides an investigative avenue from which research-based strategies to reduce hunger

during fasting periods may be elucidated. In normal weight individuals, energy homeostasis, and subsequently perceived hunger, is tightly controlled through various peripheral and central signaling factors. The hypothalamus is considered one of the central regulatory regions in this regard and responds directly to peripheral signals as well as to inputs from hindbrain noradrenergic nuclei (A1 and A2) [15]. Within the hypothalamus, the arcuate nucleus (ARC), the paraventricular nucleus (PVN), and the ventromedial nucleus (VMH) are involved in energy homeostasis [16, 17]. The ARC contains orexigenic neuron populations, including neuropeptide Y (NPY)/agouti-related protein (AgRP) expressing neurons, and anorexigenic neuron populations, including proopiomelanocortin (POMC) expressing neurons [16, 17]. The PVN contains both corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) expressing neurons. Elevations in CRH and TRH levels have been shown to decrease food intake and reduce body weight [18, 19]. Among the circulating peripheral modulators of energy homeostasis are a number of peptide hormones whose receptors can be found in these hypothalamic nuclei. One example is ghrelin, which is a gastrointestinal peptide that stimulates feeding and promotes positive energy balance [16]. Ghrelin exerts its orexigenic response by stimulating NPY/AgRP neurons and simultaneously inhibiting POMC neurons [17]. Alternatively, the adipokine leptin functions to decrease food intake by inhibiting NPY/AgRP while stimulating POMC neurons [17]. While the actions of these peripheral and central signals are well-defined in non-obese and lean animals, the role of these signals are diminished or attenuated in states of excess weight gain and obesity.

Obesity results in distinct neural and metabolic alterations that support overconsumption and weight gain [20-22]. For instance, leptin and insulin resistance, dysregulation of hypothalamic neuropeptides, and reduced satiety signals are some of the broad physiological impairments that accompany diet-induced obesity [20, 23-26]. As such, physiological changes that result in lower body weight in non-obese or lean phenotypes do not accurately represent the mechanisms of weight loss in obesity. Despite the wealth of animal studies examining how IMF improves markers for aging [27, 28], cognitive performance [29-31], and immune responses [10, 32], there are no studies in obese animals to determine how IMF promotes weight loss. An understanding of the neural and metabolic alterations that promote weight loss by IMF in obese animals can provide greater insight into developing research-based modifications to IMF protocols to reduce hunger, increase long-term compliance, and enhance maintenance of weight loss.

The goal of this study was to examine the central and peripheral changes in response to IMF in a DIO model. C57 male mice at post-natal date (PND) 49 were fed a high-fat diet (HFD; 45% fat) *ad libitum* for 8 weeks. Following this 8-week period, mice were either maintained on *ad libitum* HFD, received IMF of HFD (IMF-HFD), switched to *ad libitum* low-fat diet (LFD; 10% fat), or received IMF of LFD (IMF-LFD). While other DIO protocols have used an extended period of high-fat feeding ($\geq$ 12 weeks) [33], the rationale for the 8 week initial high fat diet feeding study was to model the target population of overweight and obese individuals that have reported the most beneficial weight loss with intermittent fasting protocols. We hypothesized that, despite being on a high fat diet, mice fed IMF-HFD would display improved glucose metabolism, enhanced

metabolic profiles, and distinct monoamine signaling in the hypothalamus comparably to LFD and IMF-LFD groups.

2.3 Materials and methods

2.3.1 Animals

Male C57BL/6 mice (n=64) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). At PND 49, all were fed an *ad libitum*, high fat diet (HFD; 4.73 kcal/g, 45% fat, 20% protein, 35% carbohydrate; D12451) for 8 weeks. Mice were then equally divided by bodyweight and transitioned to one of four experimental groups as follows: *ad libitum* HFD, IMF of HFD (IMF-HFD), *ad libitum* low fat diet (LFD; 3.85 kcal/g, 10% fat, 20% protein, 70% carbohydrate; D12450B), or IMF of LFD (IMF-LFD). All diets were obtained from Research Diets (New Brunswick, NJ). IMF mice were food deprived every other 24-hour period beginning at 9:00 AM (fasting day), 2 hours into the light cycle. On fasting days, all animals were weighed, food intake was recorded, and cages were changed. Mice were pair housed and maintained on a 12-hour light/dark cycle; lights on from 0700HR to 1900HR. All procedures were approved by the Institutional Animal Care and Use Committee of Rutgers University.

2.3.2 Body composition and RER

Body composition was assessed using the EchoMRI 3-in-1 Body Composition Analyzer (Echo Medical Systems, Houston, TX, USA) in all mice. The Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH, USA), an

indirect calorimeter, was used to measure v.O2, v.CO2, and respiratory exchange ratio (RER; v.CO2/v.O2). Mice were maintained on their respective feeding protocols and housed in the system for 48 hours, beginning on a fast day for IMF-HFD and IMF-LFD mice. The second 24-hour epoch (feeding day for IMF mice) was used for analysis.

2.3.3 Oral glucose and insulin tolerance tests

An oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT) were performed on all groups. For IMF-HFD and IMF-LFD animals, these were performed on fasting days and food was not replaced after testing. Six hours prior to the OGTT, all mice were placed in clean cages, weighed, and food deprived. At the start of the test, mice were placed in Plexiglas restrainers and a tail nick was performed to obtain a baseline glucose reading using a glucometer (AlphaTRAK 2). Immediately thereafter, mice were gavaged with a bolus of glucose (2.0 g/kg body weight) and placed in an individual clean cage without food and water. Blood samples were collected from the tail in their individual cages at 15, 30, 60, 90, 120, and 180 min post-gavage. After 180 min, all mice were returned to their home cages, water was replaced, and food was returned to HFD and LFD animals. After sufficient recovery (2-3 days), an ITT was performed after a fast in a similar manner as the OGTT with an intraperitoneal (i.p.) injection of insulin (0.75 units/kg). Blood samples were collected from the tail in their individual cages at 15, 30, 60, 90, and 120 min post-injection.

2.3.4 Plasma hormones

After a 5-hour fast (fast day for IMF-HFD and IMF-LFD), animals were euthanized by decapitation. Blood was collected; a protease inhibitor, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), at 1mg/mL was added to each sample, and samples were maintained on ice until centrifugation at 3,000 rpm for 10 min at 4°C. Plasma was stored at -80°C until analysis. Insulin, ghrelin (active), and leptin were determined by multiplex assay (EMD Millipore). A radioimmunoassay was performed to determine plasma corticosterone (sensitivity: 25 ng/ml; MP Biomedicals, Santa Ana, CA, USA) levels.

2.3.5 Biogenic amines

Brain samples were dissected from the anterior (containing the anterior hypothalamus and the paraventricular hypothalamus) and posterior portions of the medial hypothalamus (containing the arcuate nucleus and the ventromedial hypothalamus). Biogenic amines for each brain section were extracted and analyzed as previously described by reverse-phase HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific, Sunnyvale, CA, USA) with electrochemical detection (Coulochem III, Thermo Fisher Scientific) [34]. An acetonitrile-based phosphate buffer mobile phase (MD-TM; Thermo Fisher Scientific) was used for all experiments. The internal standard, 3,4-dihydroxybenzylamine (DHBA), was added to all samples prior to extraction. Quantification of norepinephrine (NE), epinephrine (EPI), dopamine (DA), and serotonin (5-HT), plus metabolites homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA),

was determined by Chromeleon 7.1 software (Thermofisher). Values were expressed as picograms (pg) divided by wet tissue weight (milligrams) of each sample.

2.3.6 Tissue dissections for qPCR

Hypothalamic nuclei were micro-dissected for RNA extraction and gene expression analysis. The PVN, ARC, and VMH were cut into 1 mm coronal slices using a brain matrix (Ted Pella, Inc., Redding, CA, USA), anterior (Bregma: -0.70 to -1.34mm) and posterior (Bregma: -1.35 to -1.94mm) [35]. The brain blocks were transferred to RNAlater (Life Technologies, Inc., Grand Island, NE, USA) and stored overnight at 4°C. Samples were dissected from slices using a dissecting microscope. Dissected tissue was stored at -80°C. Total RNA was extracted from the PVN, VMH, and ARC using Ambion RNAqueous-Micro Kits (Life Technologies, Inc). Total RNA was also DNase I-treated, using the extraction kits, at 37°C for 30 min to minimize any genomic DNA contamination. RNA quantity and quality were determined using a NanoDrop ND-2000 spectrophotometer (ThermoFisher, Inc., Waltham, MA, USA).

2.3.7 Quantitative real-time PCR

cDNA was synthesized from 200 ng of total RNA using Superscript III reverse transcriptase (Life Technologies, Inc.), 4 µL 5X Buffer, 25 mM $MgCl_2$, 10 mM dNTP (Clontech Laboratories, Inc., Mountain View, CA, USA), 100 ng random hexamer primers (Promega Corporation, Madison, WI, USA), 40 U/µL Rnasin (Promega), and 100 mM DTT in DEPC-treated water (Gene Mate, Bioexpress, Inc., Kaysville, UT, USA) in total volume of

20 µL. Reverse transcription was conducted using the following protocol: 5 min at 25°C, 60 min at 50°C, 15 min at 70°C. The cDNA was diluted to 1:20 with Nuclease-free water (Gene Mate, Bioexpress) for a final cDNA concentration of 0.5 ng/µL and stored at -20°C. Basal hypothalamus (BH) test tissue RNA was used for positive and negative controls (no reverse transcriptase) and processed simultaneously with the experimental samples.

All primers were designed to span exon–exon junctions and synthesized by Life Technologies using Clone Manager 5 software (Sci Ed Software, Cary, NC, USA). See Table 1 for a listing of all the primer sets used for quantitative real-time PCR (qPCR). For qPCR, 4 µL of cDNA template (an equivalent of 2 ng total RNA) was amplified using Sso Advanced SYBR Green (BioRad, Inc., Hercules, CA, USA) on CFX-Connect Real-time PCR instrument (BioRad). Standard curves for each primer pair were prepared using serial dilutions of BH cDNA in duplicate to determine the efficiency [$E = 10^{(-1/m)} - 1$, m = slope] of each primer pair. All efficiencies expressed as percent efficiency were approximately equal (one doubling per cycle, 90–100%). The relative mRNA expression data was analyzed using the ΔΔCT method [36, 37]. The amplification protocol for all the genes was as follows: initial denaturing at 95°C for 3 min followed by 40 cycles of amplification at 94°C for 10s (denaturing), 60°C for 45s (annealing), and completed with a dissociation step for melting point analysis with 60 cycles of 95°C for 10s, 65°C to 95°C (in increments of 0.5°C) for 5s and 95°C for 5s. The reference genes used were *Actb* and *Gapdh*. Positive and negative controls were added to each amplification run, which included a water blank. Quantification values were generated only from samples showing a single product at the expected melting point.

Final relative quantitation was done using the comparative CT method [36, 37]. The data were reported as relative mRNA expression. To determine the CT for each transcript, the threshold was consistently set at the lowest point of the exponential curve where the slope of the curve was the steepest for all plates. The relative linear quantity of target molecules was calculated using the formula $2^{-\Delta\Delta CT}$. All gene expression data were expressed as an n-fold difference relative to the HFD group.

2.3.8 Statistical analyses

The data are presented as mean ± SEM. A two-way ANOVA or two-way ANOVA with repeated measures was performed to determine schedule, diet, and diet X schedule effects. An ANCOVA with body weight as a covariate was also performed on the RER measurements [38]. A Newman-Keuls post-hoc was performed unless otherwise specified. All statistical analyses were performed using Statistica 7.1 software (StatSoft, Tulsa, OK, USA) and significance was set at $\alpha = 0.05$.

2.4 Results

2.4.1 IMF feeding reduces body weight, fat mass, and caloric intake comparably to a low fat diet

For all groups (HFD, LFD, IMF-HFD, and IMF-LFD), we measured bodyweight, food intake, and body composition. For bodyweight, there were significant effects of diet [$F(1, 28)=13.2$, $p<0.01$], schedule [$F(1, 28)=11.2$, $p<0.01$], and time X diet X schedule [$F(13, 364)=2.4$, $p<0.01$]. As demonstrated in Figure 1A, at PND133 (4 weeks following diet

interventions) the IMF-HFD and IMF-LFD groups had lower ($p<0.05$) body weights than did the HFD group. Total caloric intake was measured over the course of the experimental period. For cumulative caloric intake, there were significant effects of diet [$F(1, 12)=24.5$, $p<0.001$], schedule [$F(1, 12)=13.0$, $p<0.01$], time [$F(13, 156)=1528.4$, $p<0.001$], and time X schedule [$F(13, 156)=3.5$, $p<0.001$]. At the end of the 4 weeks, all groups had lower cumulative intakes than the HFD group ($p<0.001$), Figure 1B.

For fat mass (g), there were significant effects of diet [$F(1, 28)=18.9$, $p<0.001$], schedule [$F(1, 28)=6.8$, $p<0.05$], and diet X schedule [$F(1, 28)=13.1$, $p<0.01$]. All groups had lower fat mass than HFD group ($p<0.001$), Figure 2A. For lean mass, there was only an effect of diet [$F(1, 28)=7.8$, $p<0.01$], whereas the IMF-LFD had higher lean mass than the IMF-HFD and HFD groups ($p<0.05$ for both), Figure 2B.

2.4.2 IMF increases RER

For $v.CO_2$, there were significant effects of diet [$F(1, 28)=11.9$, $p<0.01$] and diet X schedule [$F(1, 28)=25.6$, $p<0.001$]. Both LFD and IMF-LFD groups had higher $v.CO_2$ than the HFD group ($p<0.01$ and $p<0.05$, respectively), Figure 3A. For $v.O_2$, there were significant effects of diet [$F(1, 28)=5.5$, $p<0.05$], schedule [$F(1, 28)=9.3$, $p<0.01$], and diet X schedule [$F(1, 28)=26.6$, $p<0.001$]. Additionally, $v.O_2$ was lower in the IMF-LFD group compared with all other groups ($p<0.001$), Figure 3B. Respiratory exchange ratio (RER) analysis indicated a diet effect [$F(1, 28)=243.4$, $p<0.001$] and a schedule effect [$F(1, 28)=45.1$, $p<0.001$]. RER was elevated in the IMF-HFD group relative to HFD ($p<0.001$) and lower relative to LFD ($p<0.05$), whereas the IMF-LFD group was elevated in respect to all

other groups ($p<0.001$), Figure 3C. Because body weight can influence energy metabolism, RER was analyzed by ANCOVA with body weight as a covariate. Accounting for body weight, there was a diet effect [$F(1, 27) = 209$, $p< 0.0001$] and a schedule effect [$F(1, 27) = 39.7$, $p< 0.0001$]. All groups were different from HFD ($p < 0.05$). RER was plotted as a function of body weight to illustrate the effect of diet and schedule, Figure 3D.

2.4.3 Glucose and insulin tolerances are altered by an IMF schedule of feeding

Glucose tolerance was determined over 180 minutes following an oral bolus of glucose. For glucose tolerance, there were significant effects of diet [$F(1, 28)=40.1$, $p<0.001$], schedule [$F(1, 28)=47.2$, $p<0.001$], and time X diet X schedule [$F(6, 168)=5.3$, $p<0.001$]. At 15 min, all groups had lower blood glucose levels compared with the HFD ($p<0.001$). The IMF-LFD was also lower than IMF-HFD and LFD ($p<0.001$). At 30 and 60 min, the IMF-LFD group maintained lower blood glucose than all other groups ($p<0.001$ and $p<0.05$ for all, respectively). At 90 min, IMF-LFD group had lower blood glucose levels than the HFD group only ($p<0.05$), Figure 4A. AUC analysis showed an overall reduction in oral glucose tolerance in the IMF-LFD mice compared to all other groups ($p<0.001$), Figure 4B.

Insulin tolerance was measured after an i.p. injection of insulin over 120 minutes. For insulin tolerance, there were significant effects of diet [$F(1, 28)=27.3$, $p<0.001$], diet X schedule [$F(1, 28)=9.9$, $p<0.01$], and time X diet X schedule [$F(5, 140)=10.0$, $p<0.001$]. IMF-LFD group had lower baseline glucose than all other groups ($p<0.01$), but for 60, 90, and 120 min the IMF-LFD was elevated compared with LFD group ($p<0.05$). For all time points,

except for baseline and 15 min, the LFD was lower than the HFD group ($p<0.05$). In addition, the LFD group also had lower blood glucose than the IMF-HFD group at 60, 90, and 120 min ($p<0.05$ for all), Figure 4C. AUC analysis showed a reduction as a consequence of the intermittent schedule; IMF-HFD and IMF-LFD were lower than HFD and LFD groups, respectively ($p< 0.05$ for both). Also, the LFD group had lower AUC than the HFD group ($p<0.05$), Figure 4D.

2.4.4 IMF and HFD influence terminal plasma levels of insulin and leptin but not ghrelin or corticosterone.

Plasma levels of hormones were assessed by multiplex assay. For plasma insulin levels there was an effect of diet [$F(1, 28)=5.8$, $p<0.05$]. Insulin was significantly lower in all groups compared with the HFD group ($p<0.05$), Figure 5A. Likewise, for leptin concentrations, there were effects of diet [$F(1, 28)=25.9$, $p<0.001$], schedule [$F(1, 28)=7.6$, $p<0.05$], and diet X schedule [$F(1, 28)=11.531$, $p<0.01$]. Plasma leptin were lower in all groups compared with the HFD group ($p<0.001$), Figure 5B. There were no effects of diet, schedule, or diet X schedule on terminal plasma ghrelin, Figure 5C. Similarly, we did not observe any effects on terminal corticosterone (data not shown), suggesting that the IMF protocols did not induce a stress response.

2.4.5 Medial hypothalamic norepinephrine and dopamine increase in response to IMF

Biogenic amines were measured in the anterior and posterior medial hypothalamus by HPLC. These regions are inclusive of the PVN and ARC/VMH,

respectively. For NE content in the anterior medial hypothalamus, there were effects of diet [$F(1, 27)=5.4$, $p<0.05$] and schedule [$F(1, 27)=26.0$, $p<0.001$]. There was an elevation as a consequence of the intermittent schedule. The IMF-HFD and IMF-LFD groups were higher than HFD and LFD groups, respectively ($p<0.05$ for both), Figure 6A (left). In the posterior medial hypothalamus, there were effects of schedule [$F(1, 27)=15.1$, $p<0.001$] and diet X schedule [$F(1, 27)=4.9$, $p<0.05$]. NE was increased in the IMF-LFD compared with all other groups ($p < 0.05$), Figure 6A (right). For DA content in the anterior medial hypothalamus, there were effects of diet [$F(1, 26)=18.6$, $p<0.001$], schedule [$F(1, 26)=22.1$, $p<0.001$], and diet X schedule [$F(1, 26)=4.7$, $p<0.05$]. DA concentrations were significantly higher in the anterior medial hypothalamus of IMF-LFD animals than all other groups ($p<0.001$), Figure 6B (left). For 5-HT, 5-HIAA, and HVA there were no effects of diet or schedule in either hypothalamic region, Figure 6C-E.

2.4.6 NPY and POMC mRNA expression in the ARC and adrenergic receptors in the PVN of the hypothalamus respond to an intermittent schedule of feeding.

Gene expression in the ARC, PVN, and VMH was measured by qPCR. For ARC *Npy* expression, there was an effect of schedule [$F(1, 26)=21.7$, $p<0.001$]. There was an elevation as a consequence of the intermittent schedule. The IMF-HFD and IMF-LFD groups had significantly greater *Npy* expression than both the HFD and LFD groups ($p<0.05$ for all), Table 2. For ARC *Pomc* expression, there was a diet effect [$F(1, 27)=14.8$, $p<0.001$] and schedule effect [$F(1, 27)=8.3$, $p<0.01$]. *Pomc* expression was lower in all groups compared with the HFD group ($p<0.05$), Table 2. For ARC growth hormone

secretagogue receptor (GHSR) gene expression, there was an effect of schedule [F(1, 27)=9.4, p<0.01]. The IMF-HFD and IMF-LFD groups demonstrated higher levels of *Ghsr* gene expression than the HFD group (p<0.05), Table 2. Conversely, there were no significant differences in ARC expression of *Agrp*, glucagon-like peptide 1 receptor (*Glp1r*), or the adrenergic receptors, *Adra1a*, *Adra1b*, or *Adra2c*, Table 2. In the PVN, there was an effect of schedule on *Adra1a* [F(1, 26)=8.3, p<0.01] and *Adra1b* [F(1, 26)=5.1, p<0.05], Table 3. For expression of *Adra1a*, the IMF-HFD and LFD were significantly lower than the HFD group (p<0.05). For expression of *Adra1b*, the LFD had lower levels than the HFD group (p<0.05), Table 3. Gene expressions of PVN *Adra2c*, *Crh*, *Trh,* and *Glp1r* were not significantly different between groups, Table 3. In the VMH, expressions of *Glp1r*, *Adra1a*, *Adra1b*, *Adra2b*, and *Adra2c* showed no significant effects of either diet or schedule of the diet, Table 4.

2.5 Discussion

Several clinical studies have indicated that intermittent fasting is an effective weight loss treatment for some obese and overweight populations [7, 9, 11, 39]. However, there have not been any preclinical studies examining the effects of this diet strategy in animal models of obesity. The intermittent fasting protocol used in our study was an alternate day fasting regimen with repeated 24-hour intervals of food deprivation followed by 24-hour *ad libitum* food access. Our study sought to further understand the neural and metabolic consequences of an intermittent fasting protocol in adult male DIO mice. In particular, our study promoted an obese phenotype by exposing mice to *ad*

libitum high-fat feeding for 8 weeks before beginning the intermittent fasting protocol or low fat/low calorie diet switch (LFD). One group of mice was maintained on the high-fat diet throughout the study (HFD; 12 weeks total), which was the control group in these experiments. Indeed, most intermittent fasting protocols in humans have been validated in overweight, class I obese (BMI $\leq$ 34.9), or class II obese (BMI $\leq$ 39.9) individuals [7-10, 39]. However, most of the subjects in these studies were either overweight or class I obese [39].

In order to uncover the neural and metabolic changes that promote weight loss by intermittent fasting, our measurements were taken after significant body weight loss was achieved. This was achieved at the 4 week time point in the present set of experiments. At 4 weeks, body weights were significantly lower in IMF-HFD (~13 % reduction) and IMF-LFD (~18% reduction) groups compared with the HFD group. It is important to note that all three groups (IMF-HFD, LFD, and IMF-LFD) consumed statistically similar cumulative caloric intakes over the 4-week period (~15-20% reduction compared with the HFD group). While the study did not have a pair-fed control groups, there was complete overlap in cumulative caloric intakes between the IMF-HFD and LFD diet groups (i.e., calorie-matched). As a result, there was a reduction in fat mass in all groups compared with the HFD group. This was also reflected in reduced terminal plasma leptin levels by approximately 65% in all groups compared with the HFD group. Thus, it appears that IMF of a HFD is similarly effective at reducing caloric intake and, therefore, fat accumulation, as a low fat/low calorie diet.

One interesting finding in our study was that the IMF-LFD had higher lean mass than the HFD and IMF-HFD groups. While the cause for this increase in lean mass is unknown, retention of lean mass has been reported in humans undergoing a modified intermittent fasting protocol for 7 weeks [6]. In a study by Klempel and colleagues [6], overweight or obese subjects (BMI 30-39.9; n= 32 completers) were randomly assigned to receive a high-fat diet (45% fat) or lower fat diet (25% fat). To reduce feelings of hunger, subjects were able to consume 25% of their energy needs on fast days. At the completion of the study, subjects in either the high-fat or low-fat intermittent fasting regimen lost weight from baseline (~ 4.5%), but there was no difference in amount of weight loss or body composition between diets [6]. The retention (or elevation in lean mass) is in contrast to the findings observed by Chausse and colleagues (2014) in non-obese male Sprague Dawley rats exposed to an alternate fasting protocol for 3 weeks [40]. In that study, intermittent fasting produced a reduction in epididymal fat mass, it also resulted in a reduction of soleus and plantaris muscle mass compared with *ad libitum* feeding of the same AIN-93 diet (13.8% protein, 76% carbohydrates, 10.2 % fat).

Chausse and colleagues (2014) also found similar relative RER levels between *ad libitium* and intermittent fasting (on fed days) rats [40], whereas our findings indicate an increase in RER on fed days with intermittent fasting with respect to diet (i.e., IMF-HFD was increased relative to HFD and IMF-LFD was increased relative LFD). Besides the difference in rodent species and diets, one prominent difference in the design of our study and that of Chausse et al., (2014) was that our mice were placed on high-fat diet for 8 weeks prior to the intermittent fasting regimen. Future studies will be designed to

determine if the metabolic changes associated with DIO promote the retention of lean mass during an intermittent fasting regimen.

Another major finding of the present study was that oral glucose tolerance was slightly improved with an intermittent fasting of high-fat diet. Although there was not a difference in AUC, 15 min following the oral glucose load, blood glucose levels were lower in the IMF-HFD and LFD groups. Possibly related to the retention in lean mass, the IMF-LFD had lower glucose levels, over time and when expressed as AUC, following the oral glucose load. Improvements with intermittent fasting and a LFD were also observed in the insulin tolerance tests. Notably, terminal plasma insulin levels were reduced by approximately 45% in all groups compared with the obese HFD group. Improvements in insulin levels have been noted with long-term intermittent fasting protocols in overweight and obese women [5]. In a 24-week study, overweight or obese women (30 ± 5 kg/m2 BMI) were randomly divided into either a continuous calorie restriction of 25% or an IMF protocol of 2 consecutive fasting days (75% calorie restriction) per week. At 6 months, weight reduction was 7% in both groups, but the IMF subjects had significantly improved fasting insulin (5.2 vs. 6.3 μU/ml) and lower HOMA scores (1.1 vs. 1.3 μU/mmd/L) than continuously calorie restricted subjects [5]. Reductions in plasma levels of insulin levels also have been noted with intermittent fasting protocols in non-obese rodents [27-29, 32, 41]. Therefore, combination of intermittent fasting regimen with a lower fat diet could be beneficial for the glucose homeostatic impairments associated with obesity. Because we did not measure free fatty acids, lipoproteins, or markers for fat oxidation, it is unclear

how an intermittent fasting regimen with a lower fat diet improves obesity-related impairments associated with obesity.

The increase in hypothalamic norepinephrine (NE) content as a consequence of the intermittent fasting schedule is another major finding of our study. Hypothalamic NE content was measured in the anterior section of the medial hypothalamus, inclusive of the paraventricular nucleus (PVN). In the PVN, there was a decrease in the gene expression of $\alpha 1_A$ receptor in the IMF-HFD and LFD groups, whereas the expression of $\alpha 1_B$ receptor was decreased in the LFD group compared with the HFD group. In the posterior section of the hypothalamus, inclusive of the ARC and VMH, NE content was elevated in the IMF-LFD group compared with all other groups. A predominant source of NE for hypothalamic nuclei are from a group of neurons located in caudal hindbrain (mostly the A2 cell group) [42]. Hypothalamic NE levels have been associated with the neuroendocrine response to stress and reproductive behaviors [15]. With respect to feeding behavior, elevated hypothalamic NE is critically involved in the hyperphagia that accompanies conditions of acute glucoprivation [43]. In addition, orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons in the arcuate nucleus (ARC) of the hypothalamus, which project to the PVN, are activated and NPY/ AGRP gene expression levels are increased following glucoprivation with the non-metabolized glucose analog, 2-deoxyglucose (2-DG) [44, 45]. Using immunotoxic lesions of the NE and epinephrine (E) neurons (by using saporin-conjugated to antibody for dopamine β-hydroxylase) that project to ARC, Fraley and Ritter [46] demonstrated the critical role of NE/E neurons to glucoprivic response. In particular, Fraley and Ritter found that

immunotoxin lesions of NE/E projecting neurons in the medial hypothalamus abolished the hyperphagic response to 2DG. In addition, they demonstrated NPY and AgRP basal levels were elevated, but did not alter expression levels, in response to 2DG following an immunotoxin lesion of NE/E [46]. Long-term bouts (14 days) of glucoprivation in intact animals have demonstrated the feeding response is attenuated over time, but NPY levels in the ARC remain elevated at day 14 [47]. For the reason that our measurement were taken at the 4 week time point during the initial phases of weight loss, one issue that needs examination is how *maintenance* of the weight loss achieved by intermittent fasting alters hypothalamic NE target regions. In addition, we need to determine whether elevated hypothalamic NE is directly involved with the glucose homeostasis improvements.

Findings from our study indicate that *Npy* relative mRNA levels in the ARC, similar to NE in the PVN, were elevated in response to an intermittent fasting protocol (regardless of diet) compared with HFD and LFD conditions. Although not reaching statistical significance, *Agrp* relative mRNA levels in the ARC demonstrated a similar trend as *Npy*. Interestingly, relative mRNA expression levels of the hypothalamic, anorexigenic precursor polypeptide POMC were elevated in all groups compared with HFD group. This is in agreement with previous findings that arcuate *Pomc* gene expression is reduced in animals fed a high fat diet [20] and those prone to diet-induced obesity [48]. Our findings suggest that hypothalamic NE content and *Npy* mRNA are elevated after a 5 hour fast as a consequence of the 4-week exposure to an intermittent fasting regimen. Acute bouts of food deprivation have been shown to increase hypothalamic *Npy* mRNA expression

[49, 50], an effect that may potentially be augmented by the repetitive nature of an intermittent fasting protocol. The role of NE in PVN and interactions with orexigenic peptide, NPY, are certainly mechanisms that require further attention. In addition, the elevation of *Npy* and NE as a consequence of the entrainment, not weight loss, of the intermittent feeding paradigm is another possible that needs further investigation. Because our measurements of NE content and *Npy* gene expression were performed on regions rather than distinct nuclei, future experiments will use targeted more mechanistic approaches to uncover the role of the NE and NPY in the weight loss and glucose regulatory alterations that accompany intermittent fasting.

Another finding of our study was that dopamine (DA) content in the posterior region of the medial hypothalamus was increased in the IMF-LFD compared with the IMF-HFD and LFD groups. In a study by Martin and colleagues, regional monoamine content was measured after 6 month on different dietary feeding protocols, including intermittent fasting, in male and female non-obese rats [29]. Although hypothalamic regions were not examined, there were differences in the catecholamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the cerebellum of male and female rats exposed to the intermittent fasting schedule [29]. Female rats exposed to a 40% caloric restriction for 6 months had increased performance in behavioral cognitive task which was accompanied by a decrease in DA and increase in serotonin content in the hippocampus compared with *ad libitum* fed rats [29]. Thus, the significance of the increase in DA in the posterior medial hypothalamus in DIO mice exposed to the IMF-LFD schedule is unclear. In addition, another limitation of our methods is that measurements of biogenic amine

tissue content do not provide an accurate index of biogenic amine turnover or steady-state concentrations [51].

An obstacle for intermittent fasting regimens is the feelings of hunger that accompany prolonged bouts of food deprivation [14]. Ghrelin is a gastrointestinal hormone that is elevated during period of fasting and associated with subjective feeding of hunger in non-obese adults [52]. Fasting increases GHSR in the hypothalamus [53] while diet-induced obesity via a high-fat diet has been shown to induce ghrelin resistance in arcuate NPY/AgRP neurons [54]. In addition, though the actions of peripheral ghrelin are thought to be primarily through the afferent vagal nerve to the hindbrain [55], there exist ghrelin-containing cells within the hypothalamus and specifically the ARC [56, 57]. Interestingly, hypothalamic ghrelin release decreases in glucoprivic states, such as with fasting or 2DG administration, whereas the opposite effect is observed with peripheral ghrelin release [58]. In our study, terminal ghrelin levels were not elevated, but the relative gene expression of the ghrelin receptor, *Ghsr*, was increased in the ARC of animals placed on the intermittent feeding schedules (i.e., IMF-HFD and IMF-LFD). Future work is needed to understand the feed-forward increase in *Ghsr* gene expression, subjective feelings of hunger, and the weight loss associated with intermittent fasting protocols.

Because our study design used pair-housed mice, one limitation of our findings is that diet-specific calorie restricted groups were not included in our study. Information gathered from a restricted access to diet, but not exposed to prolonged periods of food deprivation, would provide insight into whether fasting has benefits over simple calorie restriction. As it stands, our studies do not resolve the ongoing debate as to whether

intermittent fasting provides an added benefit beyond daily caloric restriction [4]. Keeping in mind the limitation of extrapolating animal studies and fasting periods to clinical practice, these studies do provide a starting point for research–based human studies to examine the efficacy of long-term intermittent fasting regimens for weight loss in certain populations of overweight or obese individuals.

2.6 Acknowledgments

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Tables and Figures

Table 1. Primer sequences used for qPCR.

Table 2 Relative gene expression in the arcuate nucleus (ARC) of DIO mice after 4 weeks on a HFD, IMF-HFD, LFD, or IMF-LFD feeding schedule (n=8/group). All gene expression data were expressed as an *n*-fold difference relative to the mean of the HFD group. (*: $p<0.05$ from HFD; #: $p<0.05$ from LFD). Data are represented as means ± SEM.

Table 3 Relative gene expression in the paraventricular nucleus (PVN) of DIO mice after 4 weeks on a HFD, IMF-HFD, LFD, or IMF-LFD feeding schedule. All gene expression data were expressed as an *n*-fold difference relative to the mean of the HFD group. (*: $p<0.05$ from HFD). Data are represented as means ± SEM.

Table 4. Relative gene expression in the ventromedial medial hypothalamus nucleus (VMH) of DIO mice after 4 weeks on a HFD, IMF-HFD, LFD, or IMF-LFD feeding schedule. All gene expression data were expressed as an *n*-fold difference relative to the mean of the HFD group. Data are represented as means ± SEM.

Figure 1. Male mice were placed on a high-fat diet (HFD; 45% Fat) to promote diet-induced obesity (DIO). After 8 weeks mice continued on the HFD (control group; n =8) were placed on an alternate day calorie deprivation intermittent fasting (IMF) protocol with HFD (IMF-HFD; n=8), switched to a control low-fat diet (LFD; 10% Fat; n=8) diet, or

placed on placed on IMF protocol with LFD (IMF-LFD; n=8). Data are represented as means ± SEM. **A:** Body weights of mice at the end of 4 weeks of the diet intervention. **B:** Average cumulative food intake (Kcal) over 4 weeks. * indicates difference ($p<0.05$) from HFD, *** indicates difference from HFD ($p<0.001$).

Figure 2. Body composition as assessed by EchoMRI in all groups at the end of 4 weeks of the diet intervention. Data are represented as means ± SEM. **A**: Fat mass (g). **B:** Lean body mass (g). * indicates difference ($p<0.05$) from HFD; $ indicates difference ($p<0.05$) from IMF-HFD.

Figure 3. Respiratory exchange ratio (RER) measured by indirect calorimetry (24 h; fed day) in all groups at the end of 4 weeks of the diet intervention. Data are represented as means ± SEM. **A:** v.CO2. **B:** v.O2 **C:** RER. **D:** RER data as a function of body weight. * indicates difference ($p<0.05$) from HFD; **indicates difference ($p<0.01$) from HFD; # indicates difference ($p<0.05$) from LFD; ### indicates difference ($p<0.001$) from LFD; $$$ indicates difference ($p<0.001$) from IMF-HFD.

Figure 4. Oral glucose and insulin tolerance tests in all groups at the end of 4 weeks of the diet intervention. Data are represented as means ± SEM. **A:** Blood glucose (mg/dl) response to an oral bolus of glucose (2 g/kg) over 180 minutes. Values for IMF-HFD and LFD overlap. **B:** Area under the curve (AUC) of glucose tolerance test. **C:** Blood glucose response to intraperitoneal injection of insulin (0.75 U/kg) over 120 minutes. **D:** AUC of

insulin tolerance test. * indicates difference ($p<0.05$) from HFD; **indicates difference ($p<0.01$) from HFD; ***indicates difference ($p<0.001$) from HFD; # indicates difference ($p<0.05$) from LFD; ### indicates difference ($p<0.001$) from LFD; ## indicates difference ($p<0.01$) from LFD; $ indicates difference ($p<0.05$) from IMF-HFD; $$ indicates difference ($p<0.01$) from IMF-HFD; $$$ indicates difference ($p<0.001$) from IMF-HFD.

Figure 5. Terminal levels of plasma hormones in all groups at the end of 4 weeks of the diet intervention. Data are represented as means ± SEM. **A**: Insulin **B:** Leptin **C:** Ghrelin. * indicates difference ($p<0.05$) from HFD; ***indicates difference ($p<0.001$) from HFD.

Figure 6. Biogenic amines were measured by HPLC in the anterior and posterior medial hypothalamus in all groups at the end of 4 weeks of the diet intervention (n=8 per group). **A:** Norepinephrine (NE). **B:** Dopamine **(**DA). **C:** Serotonin (5-hydroxytryptamine; 5-HT) **D:** 5-Hydroxyindoleacetic acid (5HIAA) **E:** Homovanillic acid (HVA) Data are represented as means ± SEM. * indicates difference ($p<0.05$) from HFD; # indicates difference ($p<0.05$) from LFD; $ indicates difference ($p<0.05$) from IMF-HFD.

Table 1.

Gene Name	Product Length	Primer Sequence	Base Pair #	Accession #
Adra1a	187	F: TCTGCTGGCTGCCATTCTTC	1638-1657	NM_013461
		R: CACTGGATTCGCAGCACATTC	1805-1824	
Adra1b	84	F: CTTCATCGCTCTCCCACTTG	1174-1193	NM_007416
		R: TAGCCCAGCCAGAACACT	1240-1257	
Adra2b	130	F: GCAGAGGTCTCGGAGCTAA	905-923	NM_009633.3
		R: GCCTCTCCGACAGAAGATA	1016-1034	
Adra2c	154	F: CTCATGGCCTACTGGTACTTC	1657-1677	NM_007418.3
		R: TGCGCTTCAGGTTGTACTC	1792-1810	
Agrp	146	F: CTCCACTGAAGGGCATCAGAA	287–307	NM_007427.2
		R: ATCTAGCACCTCCGCCAAA	414–432	
Actb	63	F: GCCCTGAGGCTCTTTTCCA	849–867	NM_007393.3
		R: TAGTTTCATGGATGCCACAGGA	890-911	
Crh	86	F: AGGAGGCATCCTGAGAGAAGT	152-173	NM_205769.2
		R: CATGTTAGGGGCGCTCTC	906-923	
Gapdh	98	F: TGACGTGCCGCCTGGAGAAA	778–797	NM_008084.2
		R: AGTGTAGCCCAAGATGCCCTTCAG	852–875	
Ghsr	122	F: CAGGGACCAGAACCACAAAC	1003-1022	NM_177330
		R: AGCCAGGCTCGAAAGACT	1107-1124	
Glp1r	190	F: TTCAAGCTGTATCTGAGCATAG	806-827	NM_021332
		R: AGATGACACGGATGAAGATAAG	974-995	
Npy	182	F: ACTGACCCTCGCTCTATCTC	106–125	NM_023456
		R: TCTCAGGGCTGGATCTCTTG	268–287	
Pomc	200	F: GGAAGATGCCGAGATTCTGC	145–164	NM_008895
		R: TCCGTTGCCAGGAAACAC	327–344	
Trh	238	F: TTCGGCTTAACGTCTTC	150-170	NM_009426.3
		R: CTTCGTCGTAACTGGTATCC	369-387	

Table 2.

Gene	HFD	IMF-HFD	LFD	IMF-LFD
Npy	0.936 ± 0.103	1.929 ± 0.269*,#	1.153 ± 0.112	2.035 ± 0.224 *,#
Pomc	1.016 ± 0.069	0.807 ± 0.063*	0.743 ± 0.055*	0.569 ± 0.073*
Agrp	1.058 ± 0.134	1.329 ± 0.189	1.004 ± 0.131	1.277 ± 0.163
Glp1r	1.060 ± 0.135	1.311 ± 0.191	1.014 ± 0.128	1.279 ± 0.167
Ghsr	1.022 ± 0.083	1.733 ± 0.171*	1.405 ± 0.204	1.744 ± 0.171*
Adra1a	1.055 ± 0.130	1.344 ± 0.199	1.010 ± 0.132	1.302 ± 0.171
Adra1b	1.058 ± 0.134	1.311 ± 0.197	1.004 ± 0.131	1.280 ± 0.168
Adra2c	1.032 ± 0.100	1.128 ± 0.237	0.945 ± 0.169	0.787 ± 0.099

NPY = neuropeptide Y; POMC = pro-opiomelanocortin; AgRP = agouti related peptide; GLP-1R = glucagon-like peptide 1 receptor; GHSR = growth hormone secretagogue receptor; Adra1A = alpha adrenergic receptor 1A; Adra1B = alpha adrenergic receptor 1B; Adra2C = alpha adrenergic receptor 2C

Table 3.

Gene	HFD	IMF-HFD	LFD	IMF-LFD
Glp1r	0.933 ± 0.138	1.060 ± 0.080	0.806 ± 0.123	0.948 ± 0.090
Adra1a	1.013 ± 0.062	0.829 ± 0.035*	0.787 ± 0.039*	0.894 ± 0.054
Adra1b	1.066 ± 0.132	0.783 ± 0.044	0.726 ± 0.066*	0.836 ± 0.065
Adra2c	1.053 ± 0.110	0.803 ± 0.082	0.892 ± 0.082	0.827 ± 0.077
Crh	1.151 ± 0.163	0.842 ± 0.143	0.994 ± 0.127	1.101 ± 0.169
Trh	1.040 ± 0.111	1.121 ± 0.146	1.091 ± 0.144	1.400 ± 0.116

GLP-1R = glucagon-like peptide 1 receptor; Adra1A = alpha adrenergic receptor 1A; Adra1B = alpha adrenergic receptor 1B; Adra2C = alpha adrenergic receptor 2C; CRH = corticotropin releasing hormone; TRH = thyrotropin-releasing hormone

Table 4.

Gene	HFD	IMF-HFD	LFD	IMF-LFD
Glp1r	1.043 ± 0.114	1.184 ± 0.100	0.930 ± 0.054	1.068 ± 0.101
Adra1a	1.003 ± 0.030	1.034 ± 0.067	0.949 ± 0.065	0.965 ± 0.052
Adra1b	1.036 ± 0.114	1.006 ± 0.066	1.031 ± 0.109	0.842 ± 0.065
Adra2c	1.018 ± 0.068	0.978 ± 0.069	1.174 ± 0.170	1.172 ± 0.165

GLP-1R = glucagon-like peptide 1 receptor; Adra1A = alpha adrenergic receptor 1A; Adra1B = alpha adrenergic receptor 1B; Adra2C = alpha adrenergic receptor 2C

Figure 1.

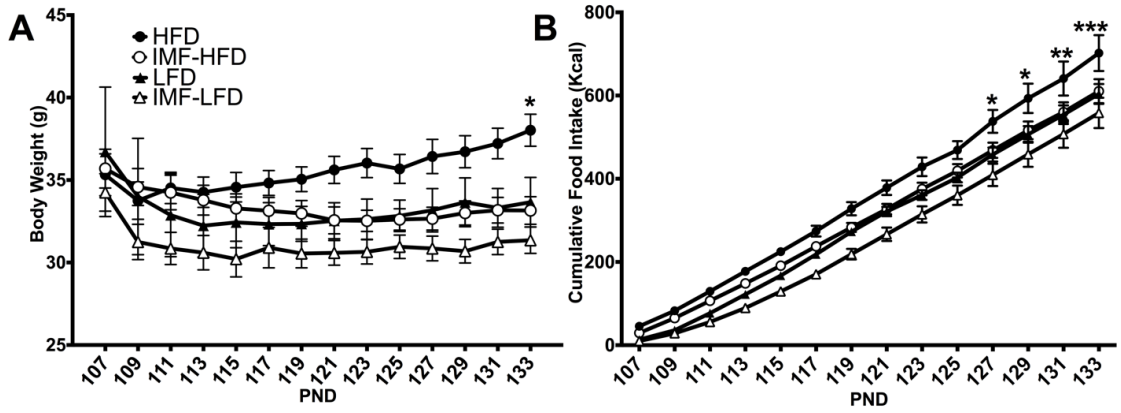


Figure 2.

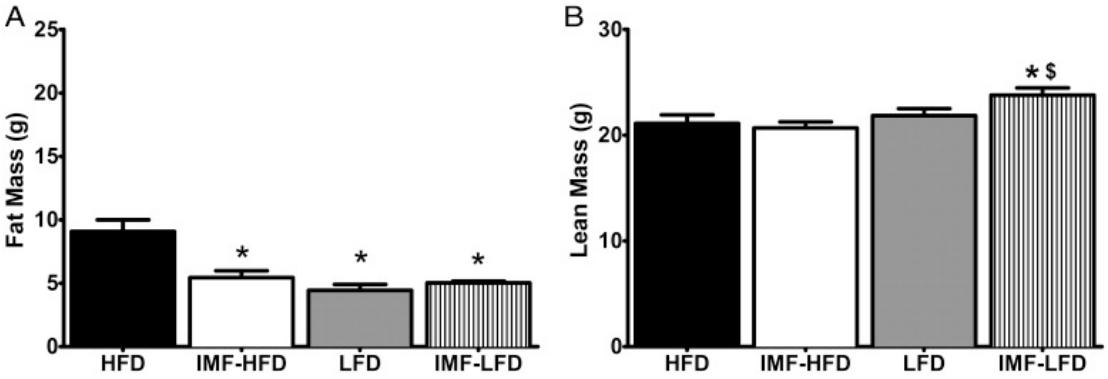
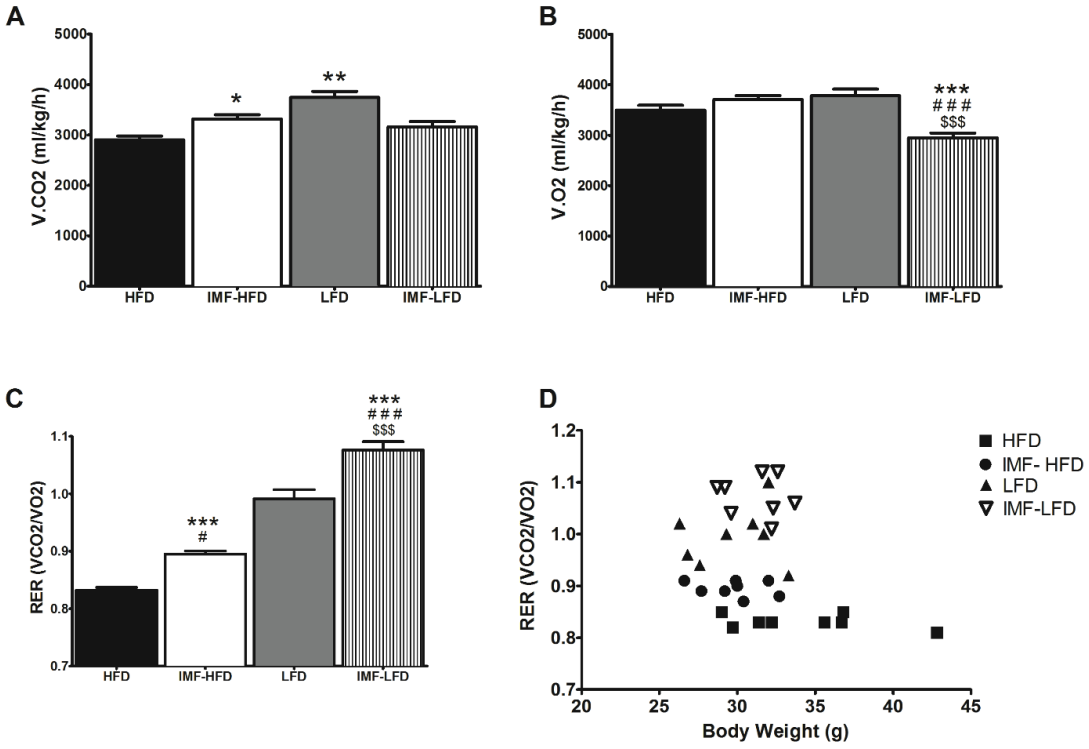


Figure 3.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V.CO2 (ml/kg/h)
5000
4000
3000
2000
1000
0
*
**
HFD
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LFD
IMF-LFD
B
V.O2 (ml/kg/h)
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###
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C
RER (VCO2/VO2)
1.1
1.0
0.9
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D
RER (VCO2/VO2)
1.2
1.1
1.0
0.9
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20
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40
45
Body Weight (g)
HFD
IMF- HFD
LFD
IMF-LFD

Figure 4.

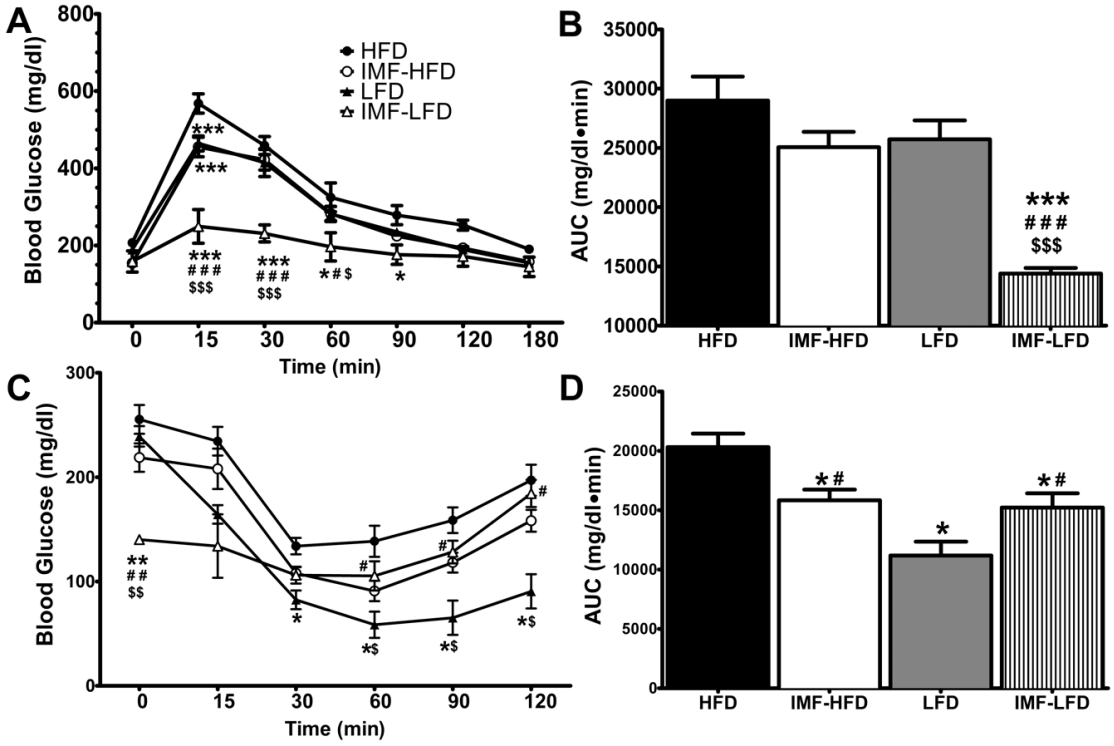


Figure 5.

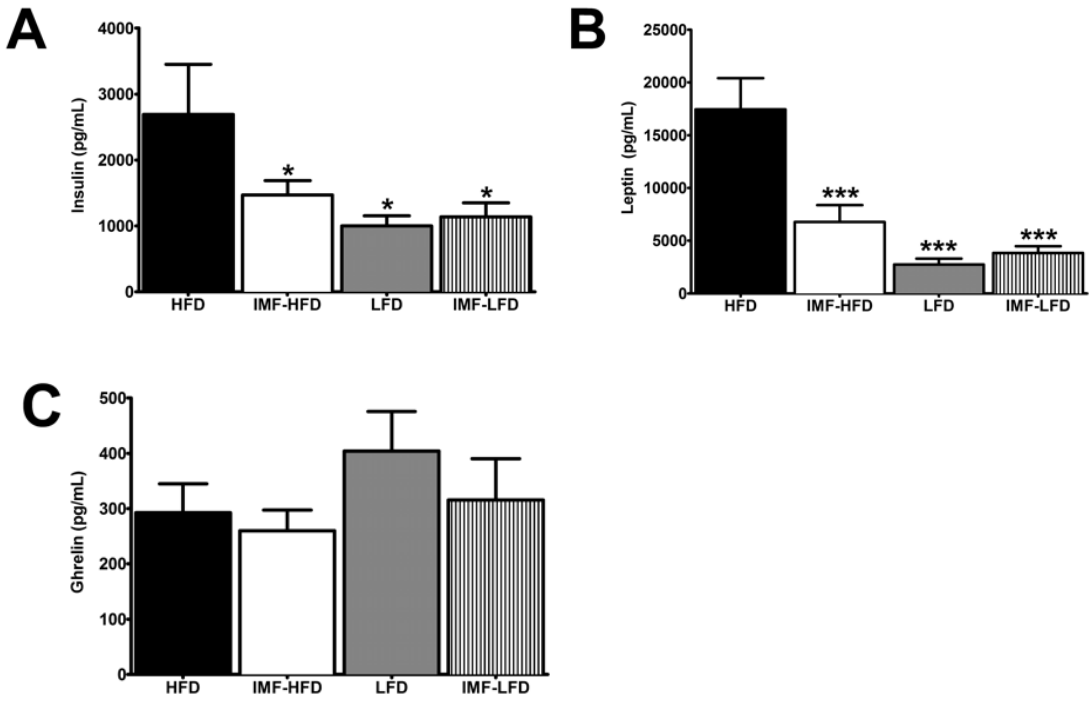
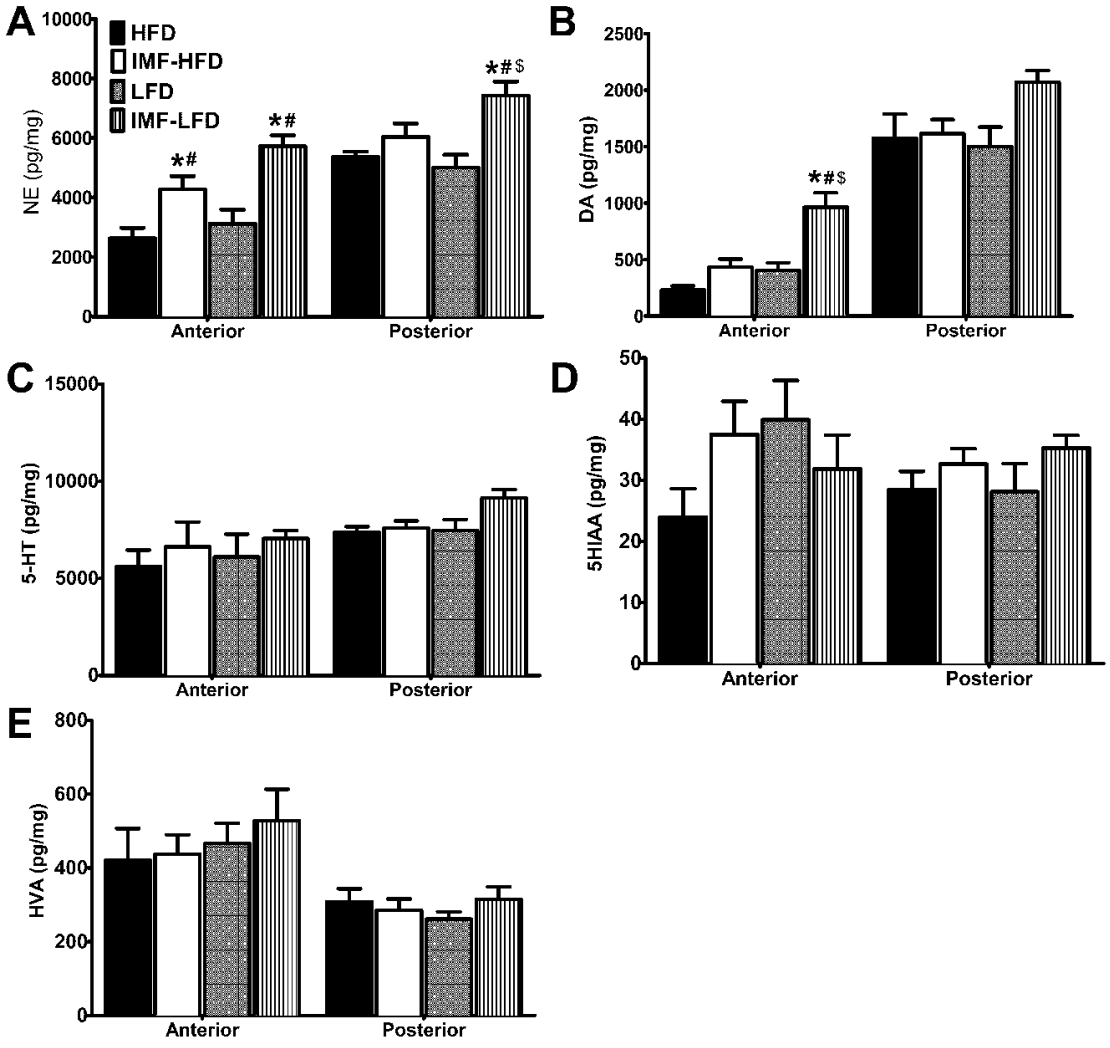


Figure 6.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IMF-LFD
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800
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Anterior
Posterior

CHAPTER 3:

Intermittent fasting does not alter neuropeptide Y neuron activation following glucoprivation in the paraventricular nucleus or arcuate nucleus of the hypothalamus in diet-induced obese male mice.

3. Intermittent fasting does not alter neuropeptide Y neuron activation following glucoprivation in the paraventricular nucleus or arcuate nucleus of the hypothalamus in diet-induced obese male mice.

3.1 Abstract

Alternate day, intermittent fasting (IMF) in diet-induced obese (DIO) mice promotes distinct neural and metabolic phenotypes compared to *ad libitum* fed controls. To further elucidate these differences, we investigated the effects of an acute glucoprivic challenge on hypothalamic neural activation in DIO mice on an IMF diet. Transgenic male mice bred to co-express green fluorescent protein (GFP) and neuropeptide Y (NPY) on a C57/BL6 background were used for this study. DIO was induced by providing *ad libitum* access to a high-fat (HFD; 45%) diet for 8 weeks. Mice were then either maintained on the HFD, given IMF of HFD (IMF-HFD), switched to a low-fat diet (LFD: 10%), or given IMF of LFD (IMF-LFD) for an additional 4 weeks. At the end of the 4 weeks, IMF-HFD (~27% reduction) and IMF-LFD (~32% reduction) weighed significantly less than HFD mice. IMF-HFD, LFD, IMF-LFD, and half of the HFD mice were given an intraperitoneal (i.p.) injection of 2-deoxy-D-glucose (2-DG) 90 min prior to sacrifice and the remainder of the HFD animals received an i.p. injection of saline. Neural activation was determined in the paraventricular nucleus (PVN) and arcuate nucleus (ARC) of the hypothalamus as counts of c-Fos positive nuclei. In the PVN, there were significantly more counts (~47%, $P<0.001$) in HFD mice given 2-DG compared to those given saline, but there were no differences in the ARC. There were no effects of dietary fat content nor schedule of access on neural activation in either region. It is possible that the IMF-

specific neural effects previously reported are due to changes in hindbrain, rather than arcuate, NPY neuron activation.

3.2 Introduction

Energy homeostasis is regulated through a delicate balance of peripheral and central circuits working in concert to prevent excessive weight fluctuations. In obesity, these circuits can be altered to promote overconsumption and weight gain, thus making it difficult to achieve and maintain weight loss [1-3]. The hypothalamus is one of the major regulatory centers of the central nervous system in this regard and responds directly to peripheral indicators of energy balance, such as insulin, leptin, and ghrelin [4, 5]. Within the hypothalamus, the arcuate (ARC) and paraventricular (PVN) nuclei play critical roles in energy homeostasis. Specifically, the ARC contains two functionally distinct neuron populations of interest – neuropeptide Y (NPY)/agouti-related protein (AGRP) containing neurons, which are orexigenic, and pro-opionmelanocortin containing neurons, which are anorexigenic [4, 5]. The PVN receives projections from ARC NPY/AGRP neurons to modulate energy homeostasis. Moreover, projections from the caudal hindbrain [A1 and A2 nuclei, which contain norepinephrine (NE)] relay information concerning peripheral peptide/hormone signals and stretch receptors from vagal afferents to the hypothalamus [6].

These NE projections are necessary to induce hyperphagia following acute glucoprivation, a state of low blood glucose [7]. 2-deoxy-D-glucose (2-DG) is a non-metabolizable glucose analog that can be injected centrally or peripherally to induce a glucoprivic state and a hyperphagic response. When these neurons are lesioned by

immunotoxin, 2-DG fails to elicit a feeding response [7]. Furthermore, lesioning results in increased NPY and AGRP gene expression in the ARC under basal conditions but completely abolishes 2-DG induced NPY and AGRP expression [8]. Previous findings from our laboratory also support a role for NE and NPY in glucoprivation induced feeding. After 4 weeks of IMF, which is characterized by alternating 24-hours of fasting induced glucoprivation, IMF mice have elevated NE content in the anterior medial hypothalamus compared to *ad libitum* fed controls [9]. IMF mice on a low fat diet also had elevated NE content in the posterior medial hypothalamus compared to all other groups [9]. Furthermore, we observed IMF specific increases in NPY gene expression in the ARC [9].

The present study aimed to evaluate neural activation in response to a glucoprivic challenge in diet-induced obese (DIO) male mice on an IMF diet. Transgenic male mice bred to express green fluorescent protein (GFP) tagged NPY were put on an *ad libitum* high fat diet at post-natal date (PND) 49 for 8 weeks. After which, animals were transition to one of four experimental groups as follows: maintained on an *ad libitum* high fat diet (HFD), received IMF of a HFD (IMF-HFD), switched to an *ad libitum* low fat diet (LFD), or received IMF of a LFD (IMF-HFD). Neural activation was assessed in the ARC and PVN following an intraperitoneal (i.p.) injection of 2-DG or saline. We hypothesized that there would be enhanced hypothalamic neural activation, particularly in NPY neurons, in animals that had received an IMF diet, thus potentially driving compensatory feeding following a fast and feelings of hunger on fasting days.

3.3 Materials and methods

3.3.1 Animals

Male transgenic mice bred to co-express GFP and NPY on a C57BL/6 background (n=41) were acquired from an established, in-house Rutgers University breeding colony. At PND 49, all were fed an *ad libitum*, high fat diet (HFD; 4.73 kcal/g, 45% fat, 20% protein, 35% carbohydrate; D12451) for 8 weeks. Mice were then transitioned to one of four experimental groups as follows: *ad libitum* HFD, IMF of HFD (IMF-HFD), *ad libitum* low fat diet (LFD; 3.85 kcal/g, 10% fat, 20% protein, 70% carbohydrate; D12450H), or IMF of LFD (IMF-LFD), see Figure 1. All diets were obtained from Research Diets (New Brunswick, NJ). IMF mice were food deprived every other 24-hour period beginning at 9:00 AM ("fasting day"), 2 hours into the light cycle. Mice remained on the experimental diets for >4 week diet period, until sacrifice. Body weight was recorded twice weekly. Mice were housed in groups of 2-3 and maintained on a 12-hour light/dark cycle; lights on from 0700HR to 1900HR. All procedures were approved by the Institutional Animal Care and Use Committee of Rutgers University.

3.3.3 Tissue collection and immunohistochemistry

All mice were food deprived for 5-6 hours beginning at 9:00 AM on the day of sacrifice. Perfusion of IMF-HFD and IMF-LFD was performed on fasting days. Mice received an intraperitoneal (i.p.) injection of 2-deoxy-D-glucose (2-DG; 500 mg/kg) 90 min prior to perfusion. The HFD group was divided into two treatment groups prior to perfusion. Half of the HFD animals received 2-DG (HFD-2DG) as described and the other half received an i.p. injection of saline (HFD-S) instead of 2-DG. Mice were deeply

anesthetized with 0.1% Euthasol (pentobarbital sodium and phenytoin sodium) solution i.p., exsanguinated with 0.9% phosphate buffered saline (PBS), and perfused with 4% paraformaldehyde in PBS. Brains were extracted and fixed for 24-hr in 4% paraformaldehyde in PBS prior to storage in a 20% sucrose/4% paraformaldehyde solution in PBS until sectioning. Free-floating hypothalamic sections (40 μm) were obtained using a Leica cryostat (Leica Microsystems, Rijswijk, The Netherlands) at -16°C and stored in cryoprotectant at -20°C until immunohistochemistry was performed [10].

For immunohistochemistry, sections were washed 3 X 10 min in PBS (10 mM phosphate, 150 mM NaCl, pH 7.5) and then blocked for 1-hour in a blocking solution [0.3% Triton X-100 (MP Biomedicals) and 0.045% normal goat serum (ThermoFisher Scientific, USA) in PBS]. After blocking, c-Fos immunolabeling was performed with a polyclonal rabbit IgG anti-human c-Fos (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA USA) primary antibody, diluted 1:000 in blocking solution. Tissue sections were incubated overnight at room temperature in the primary antibody. The next day, sections were washed 3 X 10 min in PBS then incubated for 1-hr with a fluorescently labeled secondary antibody (goat anti-rabbit IgG with Alexa Fluor 555 conjugate; ThermoFisher Scientific, USA) diluted 1:1000 in 0.3% Triton X-100 in PBS. Sections were then washed a final 3 X 10 min in PBS before mounting on gelatin-coated slides (ThermoFisher Scientific, USA). Slides were coverslipped using Prolong Diamond Antifade Mountant with DAPI (ThermoFisher Scientific, USA). All steps including and following the secondary antibody incubation were performed in the dark.

3.3.4 Fluorescent Imaging and quantification of c-Fos positive nuclei in the PVN and ARC

Imaging and quantification were performed as described elsewhere [10]. Briefly, images were captured using an Olympus FSX-BSW imaging scope and FSX100 software (Olympus Videoscope, Tokyo, Japan). Quantification was performed by identifying red-fluorescent nuclei in the PVN and ARC of the hypothalamus using the Image J software system (NIH, Bethesda, MD) image analysis software. Three anatomically matched tissue sections per mouse of each region (unilateral) were used in analysis and cells were counted by an individual blinded to the experimental treatment groups. The mean number of HFD-S counts was subtracted from each group in both the PVN and ARC to assess treatment-dependent activation only.

3.3.5 Confocal Imaging and quantification of c-Fos positive NPY neurons in the ARC

Double-labeling of c-Fos and NPY was confirmed in HFD-2-DG and HFD-S animals using a Zeiss LSM 710 (Zeiss USA) confocal microscope. Images were generated and analyzed using both Zen and Zen Lite softwares (Zeiss USA). A z-stack image was produced and quantification of double-labeling performed by identifying cells exhibiting both red (Alexa Fluor 555) and green (GFP) fluorescence. Three unilateral, anatomically matched images of the ARC were captured for each animal.

3.3.5 Statistical analyses

The data are presented as mean ± SEM. Data were analyzed by an individual two-way ANOVA or a two-way ANOVA with repeated measures to determine the effects of

schedule, diet, time, and relevant interactions. A Newman-Keuls post-hoc was performed when justified. A T-test was used to determine differences in HFD-2DG and HFD-S groups. All statistical analyses were performed using Statistica 7.1 software (StatSoft, Tulsa, OK, USA) and significance was set at $\alpha = 0.05$.

3.4 Results

3.4.1 Body weight during the 4-week diet period

Body weight (g) was measured twice weekly for all animals during the diet period. HFD-2DG and HFD-S animals were analyzed as one group as there were no significant differences between them at any time point. Over the course of four weeks, there were significant effects of schedule ($F(1, 36)=18.514$, $P<0.001$), diet ($F(1, 36)=4.3547$, $P<0.05$), time ($F(7, 252)=16.952$, $P<0.001$), time X diet ($F(7, 252)=23.383$, $P<0.001$), time X schedule ($F(7, 252)=17.716$, $P<0.001$), and time X diet X schedule ($F(7, 252)=2.9930$, $P<0.01$), see Figure 2. There was no significant effect of a schedule X diet interaction. Post-hoc analysis revealed that IMF-LFD mice achieved and maintained significantly lower body weights than HFD animals between the first and second week of the diet period, see Figure 2. Likewise, IMF-HFD mice achieved and maintained significantly lower body weights than HFD animals between the second and third week of the diet period, see Figure 2.

3.4.2 Neural activation in the PVN

Neural activation was measured in the PVN as determined by counts of c-Fos positive nuclei, see Figure 3. Glucoprivation by 2-DG administration produced a significant

increase in c-Fos positive counts in HFD-2DG animals compared to saline controls ($P<0.001$), see Figure 4. There were no significant effects of diet, schedule, or diet X schedule between HFD-2DG, IMF-HFD, LFD, and IMF-LFD groups, see Figure 4.

3.4.3 Neural activation in the ARC

Neural activation was measured in the ARC as determined by counts of c-Fos positive nuclei, see Figure 5. Glucoprivation by 2-DG administration failed to produce an increase in ARC c-fos positive nuclei, see Figure 6. There were no significant effects of diet, schedule, or diet X schedule between HFD-2DG, IMF-HFD, LFD, and IMF-LFD groups, see Figure 6.

3.4.4 Double-labeling of c-Fos and NPY in the ARC

Neural activation of NPY neurons in the ARC of HFD-S and HFD-2DG animals was determined using z-stack images produced using a confocal microscope. There were no significant differences between the groups (data not shown).

3.5 Discussion

Intermittent fasting (IMF) has recently gained popularity as a dietary strategy to reduce overall caloric intake. IMF approaches incorporate alternating periods of caloric deprivation (i.e., fasting) with those of *ad libitum* food intake (i.e., feeding) [11, 12]. One of the most frequently implemented schedules is an alternate day IMF, in which individuals alternate 24-hour periods of fasting and feeding. An intriguing facet of IMF is

that, despite an initial hyperphagic response, a total caloric deficit is typically achieved as individuals cannot completely compensate for the fasting period deficit [13]. Despite modest reductions in weight loss (3-8%) in overweight and obese individuals, feelings of hunger during the fasting period are a common barrier to long-term compliance [12-19]. Understanding the neural underpinnings of these feelings provides an opportunity to improve treatment and maintain adherence to an IMF diet. The goal of this work was to further investigate findings from Chapter 2 [9]. As such, we implemented an identical study design by promoting DIO in 7-week old male mice by providing *ad libitum* access to a high-fat diet (45%) for 8 weeks. At the end of 8 weeks, animals were either maintained on high fat diet or transitioned to IMF of HFD, a low-fat diet (10%; LFD), or IMF of LFD for 4 weeks. Concurrent with our previous findings, IMF-HFD (~27% reduction) and IMF-LFD (~32% reduction) weighed significantly less than HFD animals by the end of the dietary intervention while LFD mice did not.

2-deoxy-D-glucose (2-DG) is a non-metabolizable glucose analog. This molecule is structurally identical to glucose with the exception of a missing hydroxyl group at the 2-carbon position [20]. When 2-DG enters glycolysis, it competes with glucose for phosphorylation by hexokinase [21, 22]. Once phosphorylated, 2-DG can no longer progress through glycolysis and effectively deprives the cell of ATP – emulating a state of low blood glucose [21, 22]. Peripheral or central administration of 2-DG is followed by compensatory feeding [21, 23-28]. Following the experimental diet period, IMF-HFD, LFD, IMF-LFD, and half of the HFD animals received a glucoprivic challenge via i.p. injection of 2-DG 90 min prior to sacrifice. The remainder of the HFD group received an i.p. injection

of saline. Neural activation in the PVN and ARC of the hypothalamus was then measured in response to glucoprivation. There was an increase in c-Fos expression in the PVN as a result of 2-DG administration, but there were neither effects of schedule nor diet. Likewise, there were no effects due to glucoprivation, schedule, or diet in ARC.

Previously, we reported that IMF animals have significantly higher hypothalamic NE content and NPY mRNA expression compared to *ad libitum* fed controls, regardless of dietary fat content [9]. Furthermore, IMF animals had reduced ARC POMC mRNA expression, comparable to LFD fed controls [9]. A1 and A2 projections from the caudal hindbrain are a major source of NE in the hypothalamus [6]. Evidence from the Ritter lab supports a role for these projections in glucoprivic feeding following an injection of 2-DG [7, 29]. Indeed, when projections to the PVN are lesioned using saporin conjugated antibodies against dopamine-β-hydroxylase (DSAP), glucoprivic feeding from 2-DG is completely abolished [29]. Moreover, DSAP lesioning of hindbrain projections to the medial hypothalamus diminishes or eliminates glucoprivation-induced NPY and AGRP mRNA expression in the ARC [8, 25]. We therefore hypothesized that the repeated bouts of glucoprivation experienced during IMF promote enhanced NE signaling to the hypothalamus, subsequently increasing NPY expression and food intake.

There is evidence to suggest that hindbrain NPY neurons, rather than the downstream ARC NPY neurons, are responsible for glucoprivic feeding [23]. When ARC NPY/AGRP neurons, but not hindbrain NPY neurons, are lesioned using saporin conjugated NPY feeding response to 100, 200, and 400 mg/kg 2-DG remained intact [23]. Additionally, complete lesioning of the PVN, one of the major targets for ARC NPY/AGRP

projections, does not diminish 2-DG induced feeding [23, 24]. One limitation of this chapter is that we did not look at neural activation in the hindbrain. Though our findings do not provide an explanation for higher NE content or ARC NPY gene expression, an investigation of hindbrain NPY neuron activation in IMF animals might help elucidate some of these group differences.

Dopamine-β-hydroxylase (DBH) and tyrosine hydroxylase (TH) are commonly used markers for norepinephrine and epinephrine containing neurons [7, 30-32]. Alternatively, double-labeling for both c-Fos with DBH or TH, rather than NPY, may have shown group differences were absolute counts did not. Another limitation of this study is that we did not evaluate how 2-DG, or repeated glucoprivation through an IMF diet, affects actual feeding behavior. Meal pattern analysis during an IMF style of dieting would provide insight into the clinical relevance of the schedule specific changes we observed in chapter 2. In addition, long term studies are needed to determine if the effects of IMF on metabolic, neural, and behavioral outcomes remain after the cessation of IMF and restoration of baseline bodyweight.

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Figures

Figure 1. Experimental timeline and feeding groups. **A:** Experimental Design. All mice were placed on a 45% high fat diet for 8 weeks to promote DIO. At the end of the 8 weeks, mice were placed on one of four experimental diets for a 4-week diet period. **B:** Feeding groups. Diets and feeding conditions during the 4-week diet period.

Figure 2. Body weights over the diet period. Male mice were placed on a high fat diet to produce DIO before being transitioned to one of 4 experimental diets as follows: HFD (n=15), IMF-HFD (n=8), LFD (n=9), IMF-LFD (n = 8). Data are represented as means ± SEM. * indicates difference from HFD ($P < 0.05$). ** indicates difference from HFD ($P < 0.01$). *** indicates difference from HFD ($P < 0.001$).

Figure 3. Representative images of the paraventricular nucleus (PVN) from the HFD-2DG (top) and HFD-S (bottom) groups. **Left**: Red (Alexa Fluor 555) indicates c-Fos positive nuclei. **Center**: Green (green fluorescent protein) indicates NPY positive cells. **Right**: Overlay of c-Fos and NPY.

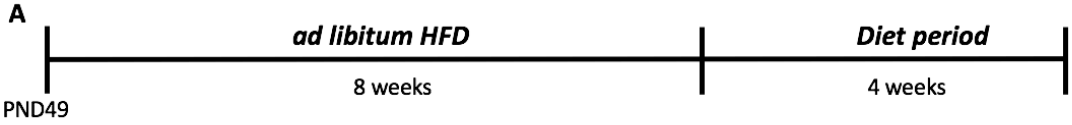
Figure 4. Counts of c-Fos positive nuclei in the paraventricular nucleus (PVN) of the hypothalamus. **A:** Average total counts in the PVN of HFD-2DG and HFD-S mice. 2-DG produced a significant increase in neural activation of the PVN. **B:** Average number saline-subtracted counts in HFD-2DG, IMF-HFD, LFD, and IMF-LFD mice. Data are represented as means ± SEM. *** indicates difference from HFD-2DG ($P < 0.001$).

Figure 5. Representative images of the arcuate nucleus (ARC) from the HFD-2DG (top) and HFD-S (bottom) groups. **Left**: Red (Alexa Fluor 555) indicates c-Fos positive nuclei. **Center**: Green (green fluorescent protein) indicates NPY positive cells. **Right**: Overlay of c-Fos and NPY.

Figure 6. Counts of c-Fos positive nuclei in the arcuate nucleus (ARC) of the hypothalamus. **A:** Average total counts in the ARC of HFD-2DG and HFD-S mice. **B:** Average number saline-subtracted counts in HFD-2DG, IMF-HFD, LFD, and IMF-LFD mice. Data are represented as means ± SEM.

Figure 1.

A



B

Group Designation	Macronutrient Composition	Kilocalorie/gram	Schedule of Diet Access
HFD	45% fat, 20% protein, 35% carbohydrate	4.73	*ad libitum*
IMF-HFD	45% fat, 20% protein, 35% carbohydrate	4.73	alternate day calorie deprivation
LFD	10% fat, 20% protein, 70% carbohydrate	3.85	*ad libitum*
IMF-LFD	10% fat, 20% protein, 70% carbohydrate	3.85	alternate day calorie deprivation

Figure 2.

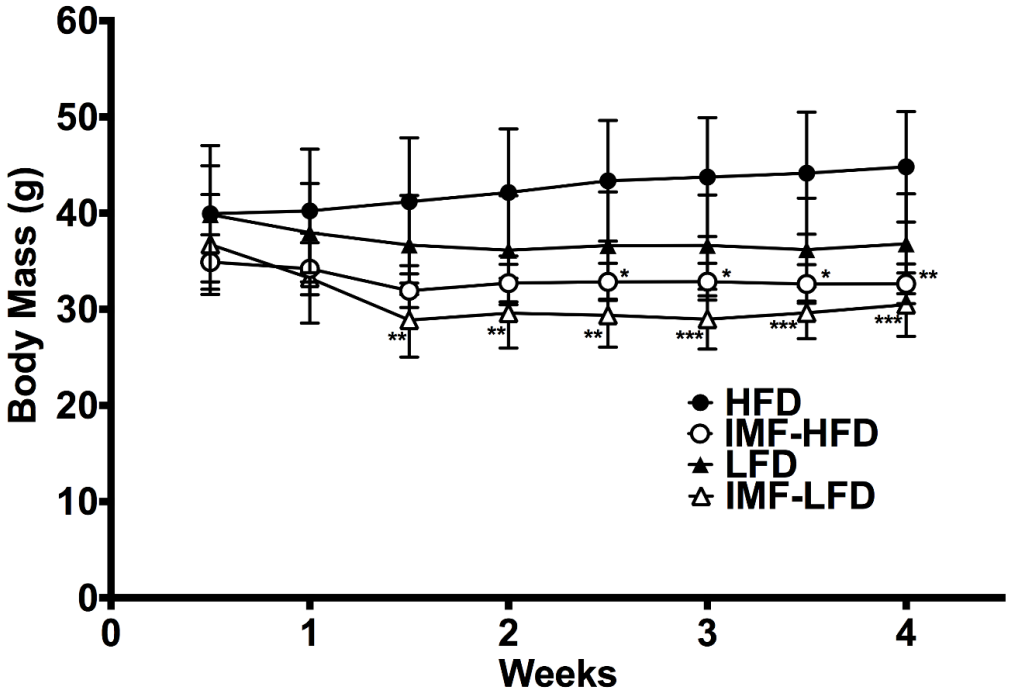


Figure 3.

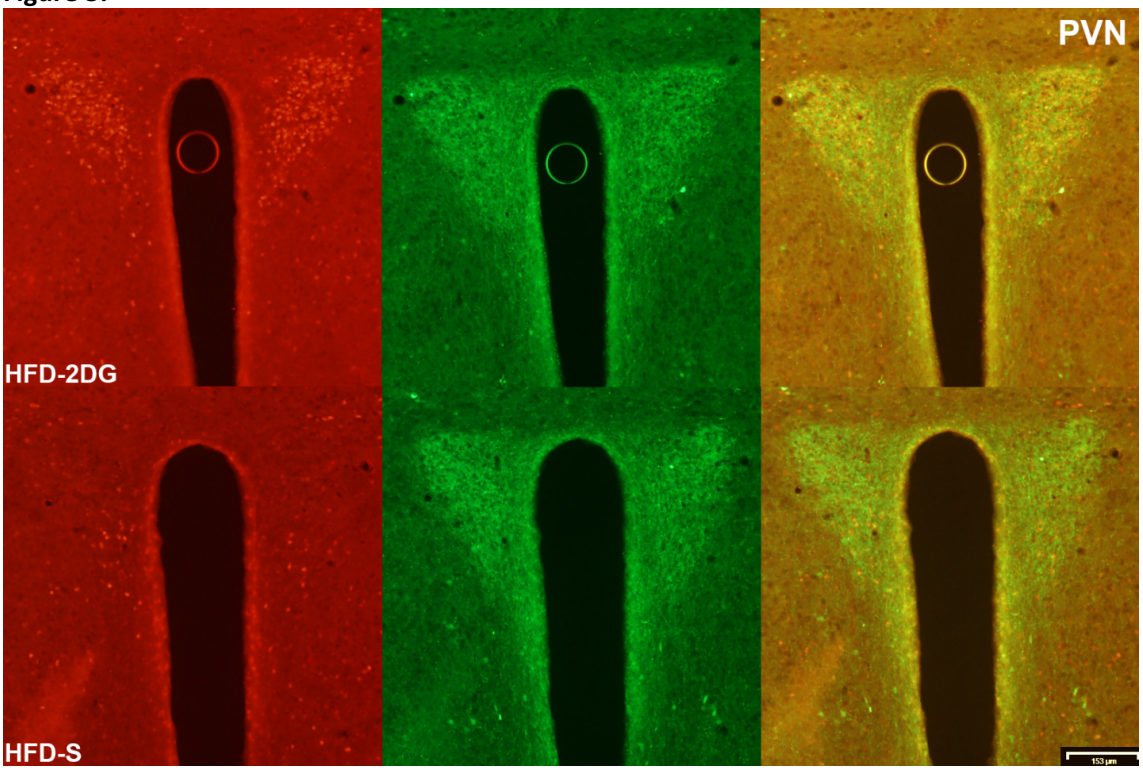


Figure 4.

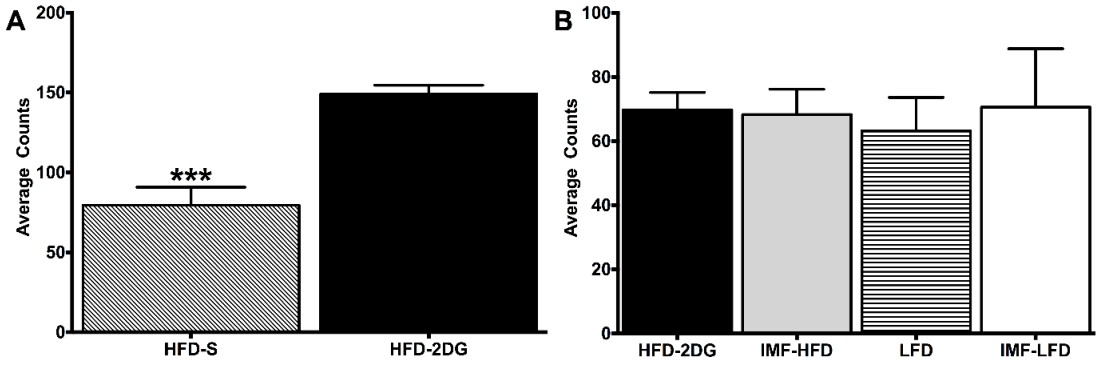


Figure 5.

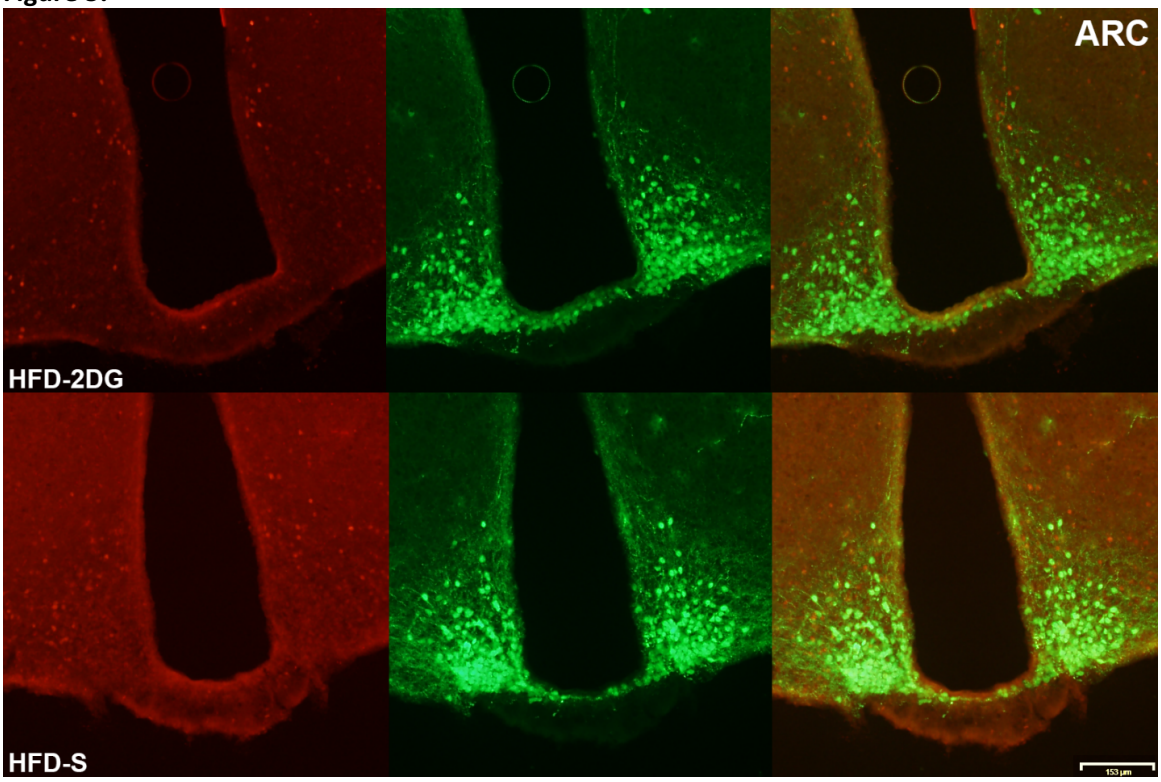
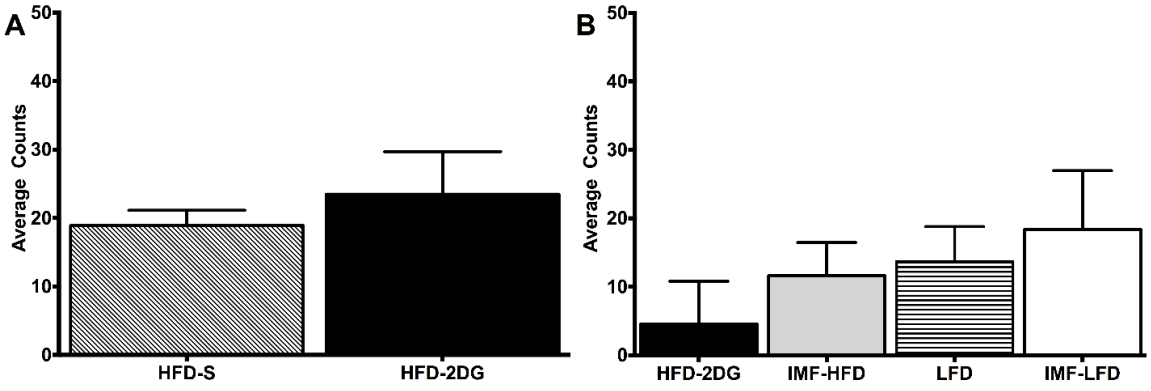


Figure 6.



CHAPTER 4:

Meal pattern alterations associated with intermittent fasting for weight loss are normalized after high-fat diet re-feeding.

4. Meal pattern alterations associated with intermittent fasting for weight loss are normalized after high-fat diet re-feeding.

4.1 Abstract

Alternate day, intermittent fasting (IMF) can be effective weight loss strategy. However, the effects of IMF on eating behaviors are not well characterized. We investigated the immediate and residual effects of IMF for weight loss on meal patterns in adult obese male C57BL/6 mice. After 8 weeks of *ad libitum* high-fat diet, mice were either continued on an *ad libitum* high-fat diet (HFD) or placed on one of 5 diet strategies for weight loss: IMF of high-fat diet (IMF-HFD), pair-fed to IMF-HFD group (PF-HFD), *ad libitum* low-fat diet (LFD), IMF of low-fat diet (IMF-LFD), or pair-fed to IMF-LFD group (PF-LFD). After the 4-week diet period, all groups were refed the high-fat diet for 6 weeks. At the end of the diet period, all 5 groups lost weight compared the with HFD group, but after 6 weeks of re-feeding with HFD, all groups had similar body weights. On (Day 2) of the diet period, IMF-HFD had a greater first meal size and faster eating rate compared with HFD. In addition, first meal duration was greater in LFD and IMF-LFD compared with HFD. At the end of the diet period (Day 28), the intermittent fasting groups (IMF-HFD and IMF-LFD) had greater first meal sizes and faster first meal eating rate compared with their respective *ad libitum* fed groups on similar diets (HFD and LFD). Also, average meal duration was longer on Day 28 in the low-fat diet groups (LFD and IMF-LFD) compared with high-fat diet groups (HFD and IMF-HFD). After 6 weeks of high-fat feeding (Day 70), there were no differences in meal patterns in groups that had

previously experienced intermittent fasting compared with *ad libitum* fed groups. These findings suggest that meal patterns were only transiently altered during alternate day intermittent fasting for weight loss in obese male mice.

4.2 Introduction

Caloric restriction (CR), either through voluntary or prescribed diets, is the most widely imposed strategy for reducing excessive body weight [1-3]. Several options exist for individuals who wish to pursue a CR diet. Daily CR typically necessitates close monitoring of caloric intake (e.g. food diaries) to achieve a 20-50% restriction from maintenance levels [4]. Another strategy, intermittent fasting (IMF), alternates periods of caloric deprivation (i.e., fasting) with periods of *ad libitum* food intake (i.e., feeding) [5]. The length of time for fasting can vary based on diet protocol, targeted clinical population, and amount of desired weight loss [4, 6]. Short term (2-24 weeks) implementation of alternate day or modified alternate day IMF effectively produces weight loss of 3-8% in both overweight and obese adult populations [6-13].

Prolonged periods of reduced calorie intake have been noted in individuals with eating disorders [14, 15]. Indeed, past prolonged bouts of CR have been associated with onset of binge eating pathologies [15-17]. In order to mimic the metabolic and motivational drive to overeat, intermittent bouts of CR are also routinely incorporated into some animal models of binge eating [18-21]. The notion that prolonged CR or IMF protocols contribute to eating pathologies, however, has not been supported by clinical studies [22, 23]. Nonetheless, several rodent studies have indicated alterations in the

neural and metabolic controls of body weight homeostasis with IMF or prolonged CR for weight loss [24-27]. One missing component to our understanding is the acute and long-term influence of calorie restricted dietary strategies on eating behaviors.

Individual meals are the functional unit of eating. The meal microstructure, which includes meal initiation, duration, and termination, are mediated through a complex balance of neural and gastrointestinal (GI) feedback controls [28, 29]. Metabolic conditions that promote weight gain, such as diet-induced obesity and leptin-deficiency, are associated with larger meal sizes [29-31]. Daily caloric intake is not only dependent on meal size, but the number of meals, and interaction between meal size and number is influenced by GI-derived peptides, such as cholecystokinin (CCK), amylin, and glucagon-like peptide-1 (GLP-1), and vagal mediated signaling [32-34]. Previous studies from our laboratory have indicated that IMF for weight loss increases NPY in the hypothalamic arcuate nucleus (ARC) and norepinephrine (NE) in the medial hypothalamus [24], which could also influence meal patterns [35, 36]. Therefore, understanding how dietary conditions, such as prolonged CR or IMF, alter meal patterns and microstructure can provide insight into the long-term controls effects of these type of dietary approaches.

The goal of the present study was to evaluate meal patterns as a consequence of IMF in diet-induced obese (DIO) male mice. As such, we compared meal patterns in DIO mice weight reduced by IMF or weight reduced by a lower calorie diet (i.e., low fat diet). The residual effects of the dietary protocols were also examined in weight restored mice. In addition, we included a continuous CR group of mice (i.e. pair-fed to IMF

groups) to determine the residual effect on meal patterns between the two different CR diet strategies. We hypothesized that weight reduction by the IMF feeding protocol would not result in persistent impairments in eating behaviors in weight restored mice. These studies are the first to investigate meal patterns during and after an alternate day intermittent fasting protocol and to determine how this type dietary strategies influences eating behaviors.

4.3 Materials and Methods

4.3.1 Animals and feeding schedules

Wild-type, male C57BL/6 mice (n = 42) were acquired from an established, in-house Rutgers University breeding colony. All mice were individually housed and maintained on a 12-hour light, 12-hour dark cycle; lights on from 7:00 AM to 7:00 PM. Mice were housed in cages that were modified for the BioDAQ system. Diets were obtained from Research Diets Inc. (New Brunswick, NJ). At PND 49, all animals were fed a high-fat diet (4.73 kcal/g, 45% fat, 20% protein, 35% carbohydrate; D12451) *ad libitum* for 8 weeks to promote diet-induced obesity (DIO). Mice were then transitioned to one of six experimental groups for 4 weeks (diet period) on either the high-fat diet or low-fat diet (3.85 kcal/g, 10% fat, 20% protein, 70% carbohydrate; D12450H) as outlined in Figure 1. IMF mice were food deprived every other 24-hour period beginning at 9:00 AM ("fasting day"). Immediately prior to fasting, all animals were weighed and food intake was recorded. Pair-fed (PF) animals were calorie matched to either IMF-HFD or IMF-LFD. That is, the PF groups received in daily calories the amount of respective diet that the

IMF-HFD and IMF-LFD consumed over the same 48 h period. The pair-feeding allotment was given at 9:00 AM daily. After the diet period, all groups were returned to, or maintained on, an *ad libitum* high-fat diet for at least 6 additional weeks (HFD re-feeding period). All procedures were approved by the Institutional Animal Care and Use Committee of Rutgers University.

4.3.2 Meal patterns and meal microstructure

Meal microstructure was analyzed using the Biological Data Acquisition system (BioDAQ; Research Diets Inc., New Brunswick, NJ). This system utilizes standard shoe-box style cages with a gated, front-mounted food hopper. The gated hopper sits upon a sensor that detects net changes in food weight. Bouts were clustered into meals, defined by an inter-meal interval of 300 seconds and a minimum of 0.02 g consumed, as recommended by Research Diets. Meal patterns were measured over the first 48 hours of the experimental diets (Days 1 & 2). That is, meal patterns were measured in IMF groups for the 24-hours food deprivation followed by 24 hours re-feeding. For the LFD groups, this was the first 48 hours of diet switch. This was repeated for the last 48 hours (Day 27 & 28) of the diet period, Figure 1. Values are reported for the second 24-hour period of each recording (i.e. the time in which IMF animals had access to food; Day 2). Meal patterns were also recorded for 24 hours at the end of the re-feeding period (Day 28), see Figure 1. PF-HFD and PF-LFD meal patterns were recorded only during the final session (post re-feeding period; Day 70). These data were used to determine the size (kilocalories; kcal), eating rate (kcal/minute), and duration (seconds) of the first meal

consumed during each recording session. Also, the average number of meals, meal size, and average duration of all meals consumed during the 24-hour recording periods were evaluated.

4.3.3 Body composition analysis, oral glucose tolerance, and insulin tolerance tests

Body composition (fat and lean mass) was measured using the EchoMRI 3-in-1 body composition analyzer (Echo Medical Systems) at the end of the high-fat diet re-feeding period (Day 70). An oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT) were also performed on all groups after the re-feeding period. The OGTT was performed approximately 1 week after the EchoMRI recordings. All animals were food deprived 5 hours before the start of the OGTT. A baseline glucose reading was measured with a glucometer (AlphaTRAK 2) via tail nick. Mice received an oral gavage of glucose (2.0 g/kg body weight) and were placed in an individual clean cage without food and water. Blood samples were collected from the tail at 15, 30, 60, 90, 120, and 180 minutes after the gavage. The test was ended at 180 minutes, all mice were returned to their home cages, water was replaced, and food was returned to all animals. After sufficient recovery of approximately 1 week (Day 84), an insulin tolerance test was performed after a 5-hour food deprivation. The ITT was conducted in a similar manner to the OGTT with an intraperitoneal injection of insulin (0.75 U/kg; Humulin Regular; rDNA origin; Eli Lilly & Company) administered just after baseline measurements were recorded. Blood samples were collected from the tail 15, 30, 60, 90, and 120 minutes

after the injection. Area under the curve (AUC) for blood glucose was calculated by the linear trapezoidal method for both OGTT and ITT.

4.3.4 Statistics

Group size was determined based on the expected variance in meal patterns using the BioDAQ system. The data are presented as mean $\pm$ SEM. Data were analyzed by an individual two-way ANOVA or by a two-way ANOVA with repeated measures for body weight, meal patterns, body composition, OGTT, and ITT outcomes. A Fisher's Least Significant Difference post-hoc testing was performed when justified. An analysis of covariance with fat mass as a covariate was also performed on the AUC OGTT measurements. All statistical analyses were performed using Statistica 7.1 software (StatSoft) and significance was set at $\alpha = 0.05$.

4.4 Results

4.4.1 Body weight and composition during the diet and HFD re-feeding periods

For all groups, we measured body weight over the course of the diet and re-feeding periods. Over the 4-week diet period, there were significant effects of diet ($F(1, 36) = 13.5$, $P < 0.001$), time ($F(14, 504) = 26.3$, $P < 0.001$), time X diet ($F(14, 504) = 16.3$, $P < 0.001$), and time X schedule ($F(28, 504) = 6.1$, $P < 0.001$), see Figure 2A. Over the course of the diet period, IMF-HFD mice received an 18.35 ± 2.63% calorie restriction compared to *ad libitum* fed HFD mice. Likewise, IMF-LFD mice received a 29.31 ± 5.91% calorie restriction compared to *ad libitum* fed HFD mice. The equivalent amount of diet

was received by the respective PF groups. By the end of the diet period, after 4 weeks, on Day 29, all diet groups (IMF-HFD, PF-HFD, LFD, IMF-LFD, and PF-LFD) weighed less than HFD mice ($P < 0.05$), see Figure 2A. For the high-fat diet re-feeding period, there were significant effects of time ($F (7, 252) = 343.7$, $P < 0.001$), time X diet ($F (7, 252) = 8.5$, $P < 0.001$), and time X schedule ($F (14, 252) = 7.2$, $P < 0.001$), see Figure 2B. There was not a diet X schedule X time effect. In addition, post-hoc testing did not reveal a difference in body weights between groups for days 42 and 49.

Body composition (fat and lean mass) was measured at the end of the HFD re-feeding period on day 70. For fat mass, there was an effect of schedule ($F (2, 36) = 6.5$, $P < 0.01$). The PF-HFD group had significantly lower fat mass than HFD ($P < 0.05$) and IMF-HFD ($P < 0.05$) groups, whereas the PF-LFD groups had significantly lower fat mass than IMF-LFD ($P < 0.05$), see Figure 2C. Similar effects were observed when fat was expressed as a percentage (%) of body weight. There were no significant differences in lean mass (g) between groups nor in body weight on the day of body composition analysis, see Figure 2D. Although all groups had a similar body weight at the end of the high-fat diet re-feeding period, the pair-fed groups demonstrated lower body fat mass than IMF groups on the same diets.

4.4.2 Oral glucose and insulin tolerance tests after 6 weeks of high-fat diet re-feeding period.

Glucose tolerance was determined over 180 minutes following an oral gavage of glucose and was performed at the end of 7 weeks of HFD re-feeding. For glucose

tolerance, there were significant effects of schedule ($F(2, 32) = 6.7$, $P < 0.01$) and time ($F(6, 192) = 97.9$, $P < 0.001$). Overall, groups that were exposed to the pair-fed schedule had lower blood glucose levels over the 180 minutes OGTT compared with IMF schedule ($P < 0.05$). Although the post-hoc testing did not reveal a difference in baseline blood glucose, there was a schedule effect ($F(2, 32) = 14.9$, $p < 0.005$) in a separate two-way ANOVA just for baseline blood glucose indicating PF groups (i.e., PF-HFD and PF-LFD) had lower blood baseline blood glucose. An AUC for blood glucose was performed for all groups. There was a significant effect of schedule ($F(2, 32) = 5.4$, $P < 0.01$) on glucose tolerance AUC. The PF-HFD and PF-LFD groups had lower AUC values compared with IMF-HFD group ($P < 0.05$ for both) for the oral glucose tolerance test, see Figure 3B. Because fat mass can influence glucose tolerance [37], AUC OGTT was analyzed by an analysis of covariance with fat mass as a covariate. Accounting for fat mass, there were no longer any overall significant effects of schedule ($F(2, 31) = 2.5$, $p = 0.10$) on AUC. Thus, the difference in fat mass, rather than schedule, accounted for the difference of OGTT on AUC values.

Insulin tolerance was determined over 120 minutes following an intraperitoneal injection of insulin. Insulin tolerance was measured at the end of the 8 weeks of HFD re-feeding. There was an effect of time ($F(5, 175) = 14.7$, $P < 0.001$) and an interaction of time X schedule ($F(10, 175) = 2.41$, $P < 0.05$) on insulin tolerance. Post-hoc testing revealed that at 15 minutes, exposure to the IMF schedule led to higher blood glucose values than groups exposed to the *ad libitum* and PF schedules ($P < 0.05$ for both). Specifically, IMF-HFD mice had significantly higher blood glucose values than HFD, LFD,

PF-HFD, and PF-LFD groups (P < 0.05 for all), see Figure 3C. There were no effects of diet, schedule or diet X schedule on AUC for the insulin tolerance test, see Figure 3D.

4.4.3 *First meal microstructure during the diet and HFD re-feeding periods.*

The first meal consumed during the recording periods were analyzed for the start and end of the diet period, as well as at the end of the re-feeding period.

4.4.4 First meal size

For first meal size (kilocalories) at the start of the diet period (Day 2), there were significant effects of diet (F (1, 24) = 14.7, P < 0.001), schedule (F (1, 24) = 28.2, P < 0.001), and diet X schedule (F (1, 24) = 23.1, P < 0.001), see Figure 4A. The IMF-HFD ate larger meals than HFD, LFD, and IMF-LFD groups (P < 0.001 for all comparisons), see Figure 4A. At the end of the diet period (Day 28), there was a significant effect of schedule (F (1, 24) = 12.4, P < 0.01). Post-hoc testing revealed that IMF-HFD and IMF-LFD ate more than the HFD (P < 0.05) and LFD (P < 0.05) groups, respectively, see Figure 4A. There were no differences between groups at the end of the HFD re-feeding period (Day 70).

4.4.5 First meal duration

For first meal duration (seconds) at the start of the diet period (Day 2), there was a significant effect of diet (F (1, 24) = 8.6, P<0.01), see Figure 4B. Post-hoc testing indicated that LFD (P < 0.01) and IMF-LFD (P < 0.05) had a longer first meal duration

than the HFD group, see Figure 4B. At the end of the diet period (Day 28), there were significant effects of diet ($F (1, 24) = 11.0$, $P < 0.01$) and schedule ($F (1, 24) = 4.85$, $P < 0.05$). Post-hoc testing revealed the IMF-LFD group ate longer meals than HFD ($P < 0.001$), IMF-HFD ($P <0.01$), and LFD ($P < 0.05$) groups, see Figure 4B. There were no differences between groups at the end of the re-feeding period (Day 70).

4.4.6 First meal eating rate

For first meal eating rate (kcal/min) at the start of diet period (Day 2), there were significant effects of diet ($F (1, 24) = 19.3$, $P <0.005$), schedule ($F (1, 24) = 14.0$, $P < 0.005$), diet X schedule ($F (1, 24) = 14.2$, $p < 0.001$), see Figure 4C. Post-hoc testing indicated that IMF-HFD had a faster eating rate than all other groups ($P < 0.05$ for all). At the end of the diet period (Day 28), there were significant effects of schedule ($F (1, 24) = 36.4$, $p < 0.0001$) and diet X schedule ($F (1, 24) = 10.1$, $p < 0.004$). Post-hoc testing indicated that IMF- HFD had a faster eating rate than all other groups ($P < 0.05$ for all) and IMF-LFD was faster than the HFD group ($P < 0.001$), see Figure 4C. There were no differences between groups at the end of the re-feeding period (Day 70).

4.4.7 Meal microstructure and patterns during the diet and HFD re-feeding periods.

Average meal size, duration, and number of meals were recorded during 24-hour recording period at the start (Day 2) and end (Day 28) of the diet period were determined and then again after the re-feeding period (Day 70).

4.4.8 Average meal size

For average meal size (kcal/meal) on Day 2, there was a significant effect of diet ($F(1, 19) = 8.6$, $P < 0.01$). Post-hoc testing revealed LFD ($P < 0.05$) and IMF-LFD ($P < 0.01$) consumed significantly smaller meal than did IMF-HFD, see Figure 5A. On Day 28, there were significant effects of diet ($F(1, 19) = 5.0$, $P < 0.05$) and schedule ($F(1, 19) = 6.2$, $P < 0.05$) on meal size. Post-hoc testing revealed IMF-LFD group had larger meal sizes than HFD ($P<0.01$) and LFD ($P<0.05$) groups, see Figure 5A. There were no significant differences in average meal size consumed after the re-feeding period.

4.4.9 Average meal duration

For meal duration (seconds) on Day 2, there was a significant effect of diet ($F(1, 19) = 5.0$, $P < 0.05$). Post-hoc testing revealed the LFD group exhibited significantly longer average meal durations than HFD and IMF-HFD groups ($P < 0.05$ for both), see Figure 5B. On Day 28, there was a significant effect of diet ($F(1, 19) = 41.9$, $P < 0.001$) on meal duration. Post-hoc testing indicated that LFD and IMF-LFD exhibited longer meals than HFD and IMF-HFD ($P < 0.001$ for all), see Figure 5B. On Day 70, there was an effect of diet on meal duration ($F(1, 36) = 5.83$, $P < 0.05$). Post-hoc testing indicated IMF-LFD consumed shorter meals than HFD and IMF-HFD ($P < 0.05$), see Figure 5B.

4.4.10 Average meal number

On Day 2, there was an effect of diet ($F(1, 19) = 26.2$, $P < 0.001$) on meal number. Post-hoc testing indicated that LFD groups consumed significantly fewer meals

than HFD ($P < 0.01$) and IMF-HFD ($P < 0.001$) groups, see Figure 5C. Further, the IMF-LFD group also consumed significantly fewer meals than HFD ($P < 0.05$) and IMF-HFD ($P < 0.001$) groups. There were no significant differences in meal numbers consumed at the end of the diet period on Day 28 and re-feeding periods on Day 70, see Figure 5C.

4.4.11 Caloric intake on meal microstructure and patterns recording days during the diet period.

Total caloric intake for the first 48 h of the diet period, Days 1-2, was measured for HFD, IMF-HFD, LFD, and IMF-LFD groups during meal patterns recording days. There were significant effects of diet ($F (1, 19) = 129.8$, $P < 0.0001$), schedule ($F (1, 19) = 16.2$, $P < 0.001$), and diet X schedule ($F (1, 19) = 10.4$, $P < 0.005$). Post-hoc testing revealed that HFD mice consumed significantly more kilocalories than all other groups ($P < 0.001$ for all comparisons) and that IMF-HFD mice consumed significantly more kilocalories than LFD and IMF-LFD mice ($P < 0.001$ for all comparisons), see Figure 6A.

For the end of the diet period, Days 27-28, there were no significant effects of diet, schedule, or an interaction effect of diet X schedule on the total caloric intake for days 27-28 at the end of the diet period, see Figure 6B.

4.5 Discussion

This study sought to investigate the effects of alternate day IMF on feeding behavior in adult DIO male mice on an IMF protocol and after a re-feeding recovery period. A DIO phenotype was induced by placing all mice on an *ad libitum* high fat (45%) diet for 8 weeks. Mice were then sorted into one of 5 dietary protocols or continued on the high fat diet (control) for an additional 4 weeks (i.e., diet period). For this study, we used a factorial design that examined the feeding schedule (IMF vs. *ad libitum*), diet (45% fat vs. 10% fat), and the interaction of schedule and diet. At the start of the diet period on Day 2 (i.e., after a 24 h food deprivation), an analysis of meal patterns indicated there was a significant increase in the size (kcal) of the first meal and increase in first meal eating rate in the IMF-HFD group. Day 2, in fact, was the second day of the LFD and the first day IMF-LFD group were exposed to the novel 10% fat diet. As a result, first meal and average meal duration was longer in the IMF-LFD compared with IMF-HFD and HFD. In addition, the caloric intake over the first 48 h, days 1-2, of the LFD and IMF-LFD groups were considerably less than the IMF-HFD and HFD groups.

By the end of the diet period, Day 28, all groups had lost body weight compared with HFD group. In addition, both the IMF-HFD and IMF-LFD groups consumed a significantly larger first meal and had a higher first meal eating rate suggesting that the alternate day IMF schedule increases first meal parameters, regardless of diet. The IMF-LFD also had a longer first meal duration compared with all other groups, which was a likely consequence of the lower calorie density of the 10% fat diet and needing a longer duration to achieve the same level of caloric intake as the IMF-HFD group. The lower

calorie density of the 10% fat diet could also explain the larger average meal size by the IMF-LFD group compared with HFD. Indeed, over the 48-h period on days 27-28, the calorie intake was similar between all groups. These alterations in meal microstructure and patterns produced by the IMF schedule did not persist when animals were returned to high-fat diet for 6 weeks and restored to a similar body weight as the HFD group on day 70. Interestingly, the group previously exposed to the IMF-LFD schedule had a shorter average meal duration than the *ad libitum* HFD and the previously exposed IMF-HFD groups after 6 weeks on the HFD. The shorter meal duration did not have an overall influence on the meal patterns since there were not any significant differences in other meal parameters. Taken together, our findings suggest that IMF schedule alters first meal parameters (i.e., size, duration, and rate), but other meal factors (i.e., average meal size, and average meal duration) were more influenced by the diets (45% fat vs. 10% fat) during the diet period.

Similar to our present findings and using an identical study design, we previously demonstrated that alternate day IMF protocol (IMF-HFD and IMF-LFD) and 10% fat diet (LFD) in obese mice produced a reduction in body weight after 4 weeks compared with HFD group [24]. In that study, the diet groups also had a reduction in cumulative calories, lower fat mass, reduced plasma insulin, reduced plasma leptin, lower AUC glucose in response to ITT, and lower ARC gene expression of POMC compared with HFD group [24]. Also similar to the present study, there were differences between the IMF compared with *ad libitum* fed groups. In particular, we previously observed that the IMF groups had increased RER (v. CO_2/v.O_2), increased NE in the anterior medial

hypothalamus, and increased ARC gene expression of NPY compared with respective *ad libitum* fed groups on the same diet [24].

NPY projections from the ARC to the parvocellular division of the hypothalamic paraventricular nucleus (PVN) have been shown to be involved in macronutrient selection using an *ad libitum* feeding paradigm in rats [38]. Specifically, exogenous injections of NPY (1-36) into the PVN have been shown to selectively increase carbohydrate intake by increasing meal sizes [35]. In addition, an increase in average meal number has been demonstrated when NPY is overexpressed in the PVN, whereas meal sizes, but not meal number, were increased when NPY was overexpressed in the lateral hypothalamic area [39]. Increases in hypothalamic NE also have shown to increase meal sizes. Exogenous injections of NE into the PVN increases first meal size after injection, eating rate, and average meal sizes of standard diet sweetened with 20% sucrose in rats [36]. Based on previous findings that the IMF protocol increases hypothalamic NPY and NE [24], it could be speculated that our observed increased first meal size and eating rate as a result of the alternate day IMF protocol could be a consequence of an increased in hypothalamic NPY and NE signaling. This notion, however, needs to be verified with additional experiments.

In order to address whether the pattern of alternate day intermittent fasting or the reduction in cumulative calories produced alterations in meal patterns, we included a pair-fed control group (i.e., PF- HFD and PF-LFD) for each IMF group (i.e., IMF-HFD and IMF-LFD) in this study. These PF groups received a daily allotment of diet, which was the averaged 48 h intake (i.e., 24 h fasted/24 h re-fed) by the respective IMF groups. Similar

to their respective IMF groups, the PF-HFD group received an approximate 18% calorie restriction and the PF-LFD received an approximate 29% calorie restriction compared to *ad libitum* fed HFD group over the diet period. One major limitation of for the PF groups was that we were unable to accurately obtain meal patterns and microstructure of meals during the diet period. The major technical issue was that the PF groups, frequently pulled food pellets into their cages, skewing the meal pattern analysis towards significantly larger meals of shorter duration. As a result, the PF groups were dropped from the diet period analyses. Another limitation was that the pair-feeding schedule likely imposed a variable period of daily fasting. Although the time after feeding was not recorded when the PF group consumed their daily allotment, the PF groups rarely had any remaining food in their cages the following day. Hence, the PF groups most likely had some period of daily intermittent fasting. In this study, after $\geq$ 6 weeks on the HFD, the previously exposed PF groups had a lower blood glucose AUC following the oral glucose tolerance test. The previously exposed PF groups also had lower fat mass. The lower fat mass accounted for the lower blood glucose AUC [40], but it is unclear why the previous exposed PF groups had lower fat mass after 6 weeks on the HFD. Despite no difference in body weight between the groups on day 78, after 49 days on re-feeding of HFD, one possibility is that PF groups had a different trajectory in body composition restoration compared with the other diet groups. A previous study by Seimon and colleagues [27] examined weight regain following weight loss in DIO C57BL/6 mice with intermittent diet (cycled ~18% calorie restriction for 5-6 days) and restricted continuous (~18 %) diet. During the weight regain phase (3 weeks) mice were

re-fed chow and despite no differences in body weight between the groups, the two previous DIO groups exposed to an intermittent diet or continuous restriction had higher fat mass than the lean ("never obese") chow-fed controls [27]. Moreover, the intermittent diet group had increased epididymal fat compared lean chow-fed after weight regain, an effect that was not observed in the continuous restricted group [27]. One notable difference between Seimon and colleagues study and the present study is that we exposed the mice to HFD following the diet period. Nonetheless, the Seimon and colleagues study illustrates how body fat can be differential effected during weight regain following weight loss.

One overall limitation of this study is that we performed the study in males. We know that there are sex-dependent differences in food intake and weight loss [41], however, we would expect to find similar changes in the meal patterns during the diet period. An expected sex-dependent difference would likely influence the time to weight loss and recover of body weight following the re-feeding of the high-fat diet [41]. Nonetheless, this study was the first to characterize eating patterns in an alternate day intermittent fasting protocol for weight loss in obese male mice. The major finding that there were no residual effects on meal patterns following weight restoration suggests the effects on eating are transient. In addition, we anticipate that neural and metabolic effects previously characterized by our lab and others [24-27] are lost with weight restoration, but this remains to be determined with future experiments.

4.6 Acknowledgments

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Figures

Figure 1. Experimental timeline and feeding groups. **A:** Experimental Design. All mice were placed on a 45% high fat diet for 8 weeks to promote DIO. At the end of the 8 weeks, mice were placed on one of six experimental diets for the 4-week diet period. Feeding behavior was measured at the start and end of the diet period. In week 12, all mice regardless of experimental diet, were transitioned back to an *ad libitum* 45% high fat diet for a 6 week re-feeding period. Feeding behavior was also measured at the end of the 6 week re-feeding. **B:** Feeding groups. Diets and feeding conditions during the 4-week diet period.

Figure 2. Body weights over the experimental periods and body composition after 6 week high-fat diet re-feeding. Male mice were placed on a high fat diet to produce DIO before being transitioned to one of 6 experimental diets (HFD, IMF-HFD, PF-HFD, LFD, IMF-LFD, PF-LFD; n = 6-8) during the diet period. At the end of the diet period, mice were again placed on a high fat diet for a 6-week high-fat diet re-feeding period. Data are represented as means ± SEM. **A:** Body weights (g) for all groups during the diet period. **B:** Body weights during re-feeding for all groups previously exposed the respective protocol. All groups were fed the high-fat diet during the re-feeding period. Body composition was assessed by EchoMRI in at the end of the 6-week re-feeding period. **C:** Fat mass (grams). **D:** Lean body mass (grams). ¥ indicates difference from all other groups ($P < 0.05$). * indicates difference from HFD ($P < 0.05$). + indicates

difference from IMF-HFD (P < 0.05). ^ indicates difference from IMF-LFD (P < 0.05). ^^ indicates difference from IMF-LFD (P < 0.01).

Figure 3. Oral glucose tolerance (OGTT) and insulin tolerance (ITT) tests following 6 weeks *ad libitum* high-fat feeding. Data are represented as means ± SEM. **A:** Blood glucose (milligrams/deciliter) response to an oral gavage of glucose (2 g/kg) over 180 minutes. **B:** Area under the curve (AUC) of OGTT. **C:** Blood glucose response to an intraperitoneal injection of insulin (0.75 U/kg) over 120 minutes. **D:** AUC of ITT. + indicates difference from IMF-HFD (P < 0.05). $ indicates difference from HFD, LFD, PF-HFD, PF-LFD (P < 0.05).

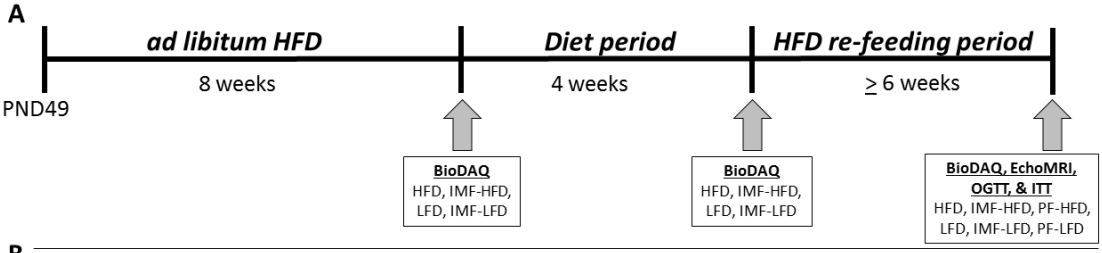
Figure 4. First meal microstructure at the initiation and termination of 4 week diet period and following 6 week *ad libitum* high-fat feeding. First meal consumed of the recording periods were evaluated at the start of the diet period (Day 2), the end of the diet period (Day 28), and at the end of the re-feeding period (Day 70). Data are represented as means ± SEM. Comparisons are between groups within each day. PF-HFD and PF-LFD meal patterns were recorded at the end of the re-feeding period (Day 70). **A:** Meal size (kilocalories), **B:** Meal duration (seconds), and **C:** Eating rate (kcal/min). * indicates difference from HFD (P<0.05). ** indicates difference from HFD (P < 0.01). *** indicates differences from HFD (P < 0.001). + indicates difference from IMF-HFD (P < 0.05). ++ indicates difference from IMF-HFD (P < 0.01). +++ indicates difference from IMF-HFD (P < 0.001). # indicates difference from LFD (P < 0.05).

Figure 5. Average meal microstructure and meal patterns at the initiation and termination of 4 week diet period and following 6 week *ad libitum* high-fat feeding. Meal patterns consumed during the 24-hour recording periods were evaluated at the start of the diet period (Day 2), the end of the diet period (Day 28), and at the end of the re-feeding period (Day 70). Data are represented as means ± SEM. Comparisons are between groups within each day. PF-HFD and PF-LFD meal patterns were recorded at the end of the re-feeding period (Day 70). **A:** Meal size (kilocalories), **B:** Meal duration (seconds), and **C:** Meal number. * indicates difference from HFD ($P < 0.05$). ** indicates difference from HFD ($P < 0.01$). *** indicates difference from IMF-HFD ($P < 0.001$). + indicates difference from IMF-HFD ($P < 0.05$). ++ indicates difference from IMF-HFD ($P < 0.01$). +++ indicates difference from IMF-HFD ($P < 0.001$). ^ indicates difference from HFD and IMF-HFD ($P < 0.05$). # indicates difference from LFD ($P < 0.05$).

Figure 6. Caloric intake during the meal microstructure and meal patterns measurements at the initiation and termination of 4 week diet period. Caloric intakes during the 48-hour recording periods were evaluated at the start of the diet period (Day 1- 2) and the end of the diet period (Day 27-28). Data are represented as means ± SEM. **A:** Days 1-2, and **B:** Days 27-28. ¥¥¥ indicates difference from all other groups ($P < 0.001$).

Figure 1.

A



B

Group Designation	Macronutrient Composition	Kilocalorie/gram	Schedule of Diet Access
HFD	45% fat, 20% protein, 35% carbohydrate	4.73	*ad libitum*
IMF-HFD	45% fat, 20% protein, 35% carbohydrate	4.73	alternate day calorie deprivation
PF-HFD	45% fat, 20% protein, 35% carbohydrate	4.73	Pair fed (to IMF-HFD)
LFD	10% fat, 20% protein, 70% carbohydrate	3.85	*ad libitum*
IMF-LFD	10% fat, 20% protein, 70% carbohydrate	3.85	alternate day calorie deprivation
PF-LFD	10% fat, 20% protein, 70% carbohydrate	3.85	Pair fed (to IMF-LFD)

Figure 2.

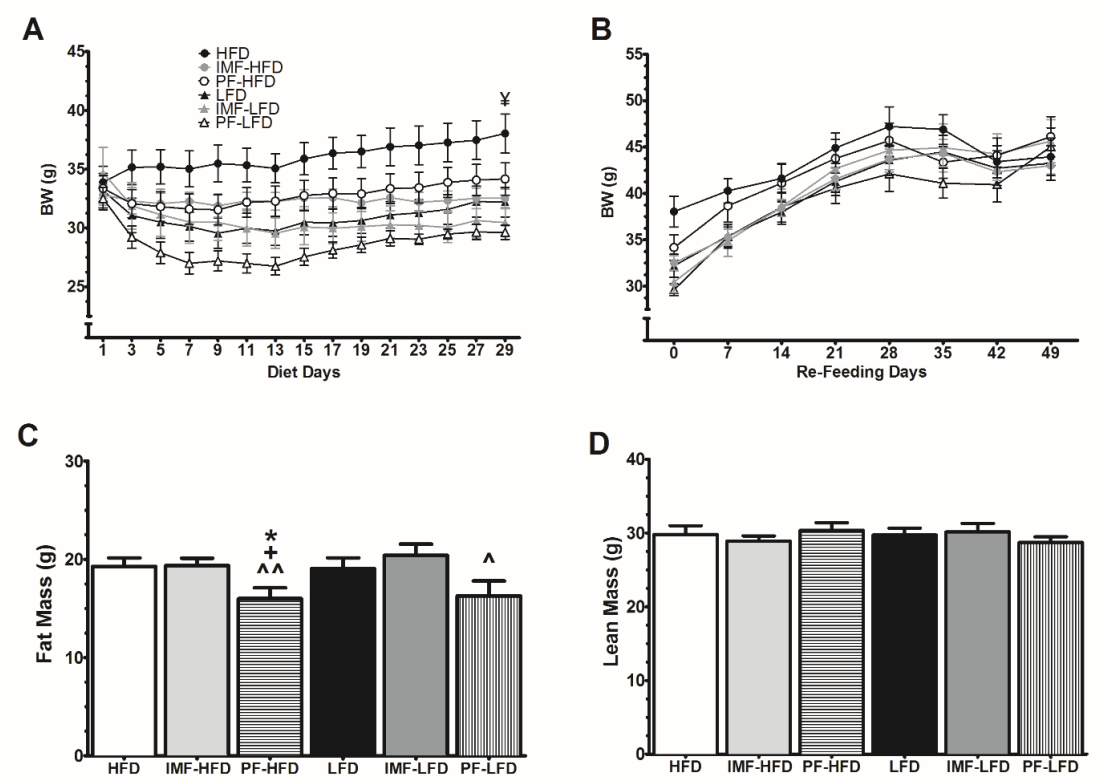

A
HFD
IMF-HFD
PF-HFD
LFD
IMF-LFD
PF-LFD
¥
BW (g)
Diet Days
B
BW (g)
Re-Feeding Days
C
Fat Mass (g)
*
+
^^
^
HFD
IMF-HFD
PF-HFD
LFD
IMF-LFD
PF-LFD
D
Lean Mass (g)
HFD
IMF-HFD
PF-HFD
LFD
IMF-LFD
PF-LFD

Figure 3.

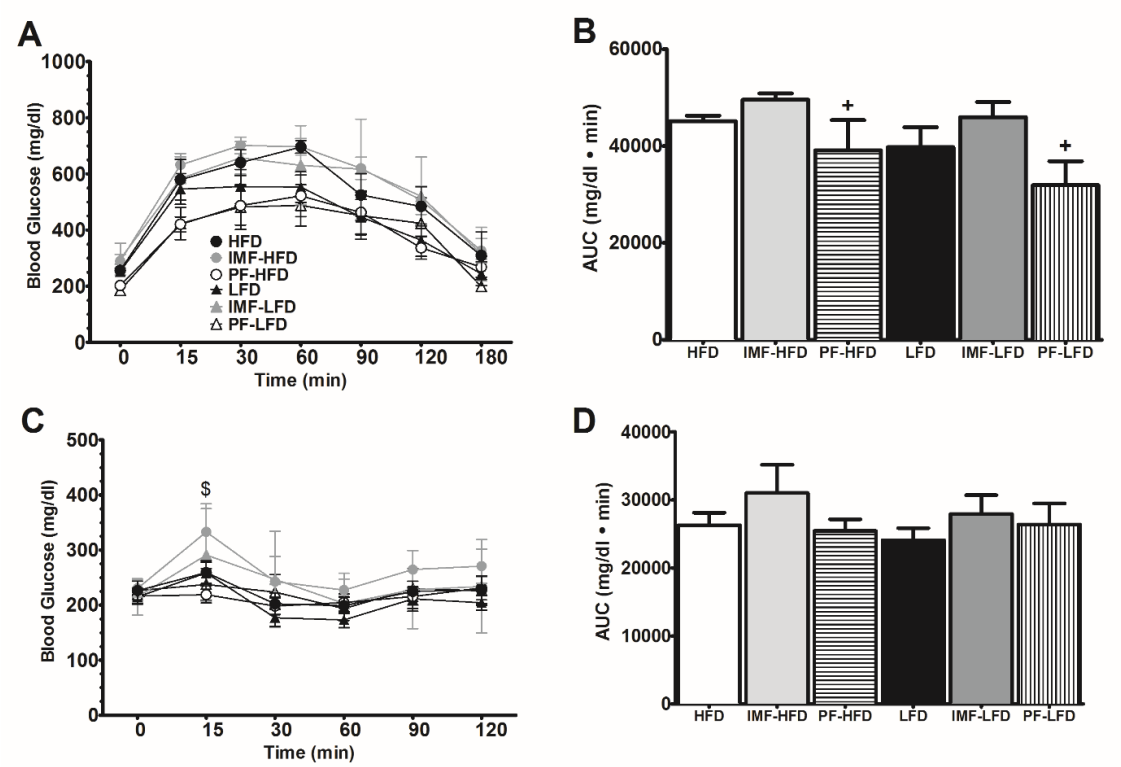

A
Blood Glucose (mg/dl)
Time (min)
HFD
IMF-HFD
PF-HFD
LFD
IMF-LFD
PF-LFD
B
AUC (mg/dl • min)
C
Blood Glucose (mg/dl)
$
Time (min)
D
AUC (mg/dl • min)

Figure 4.

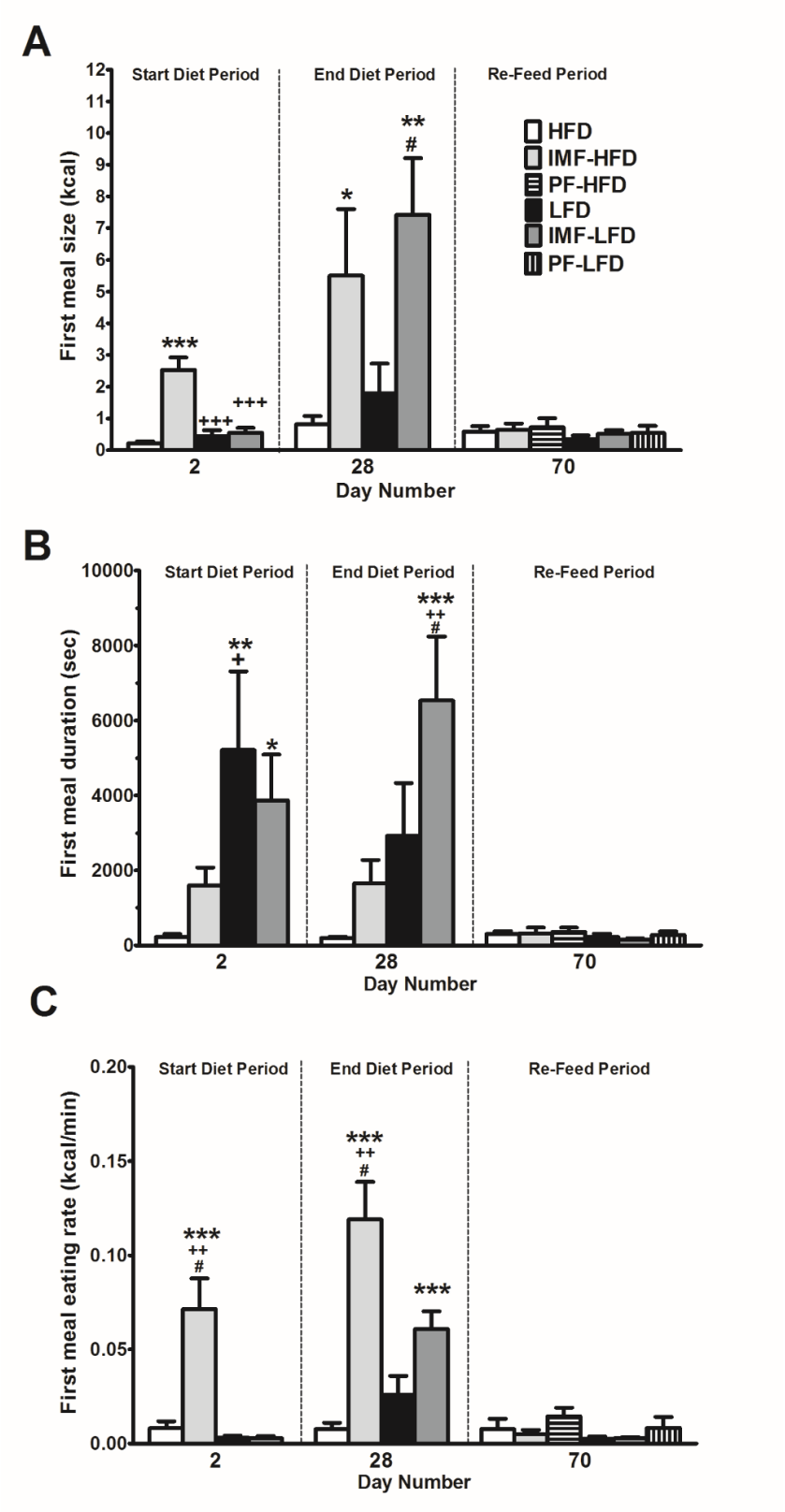

A
Start Diet Period
End Diet Period
Re-Feed Period
First meal size (kcal)
HFD
IMF-HFD
PF-HFD
LFD
IMF-LFD
PF-LFD
Day Number
B
First meal duration (sec)
Day Number
C
First meal eating rate (kcal/min)
Day Number

Figure 5.

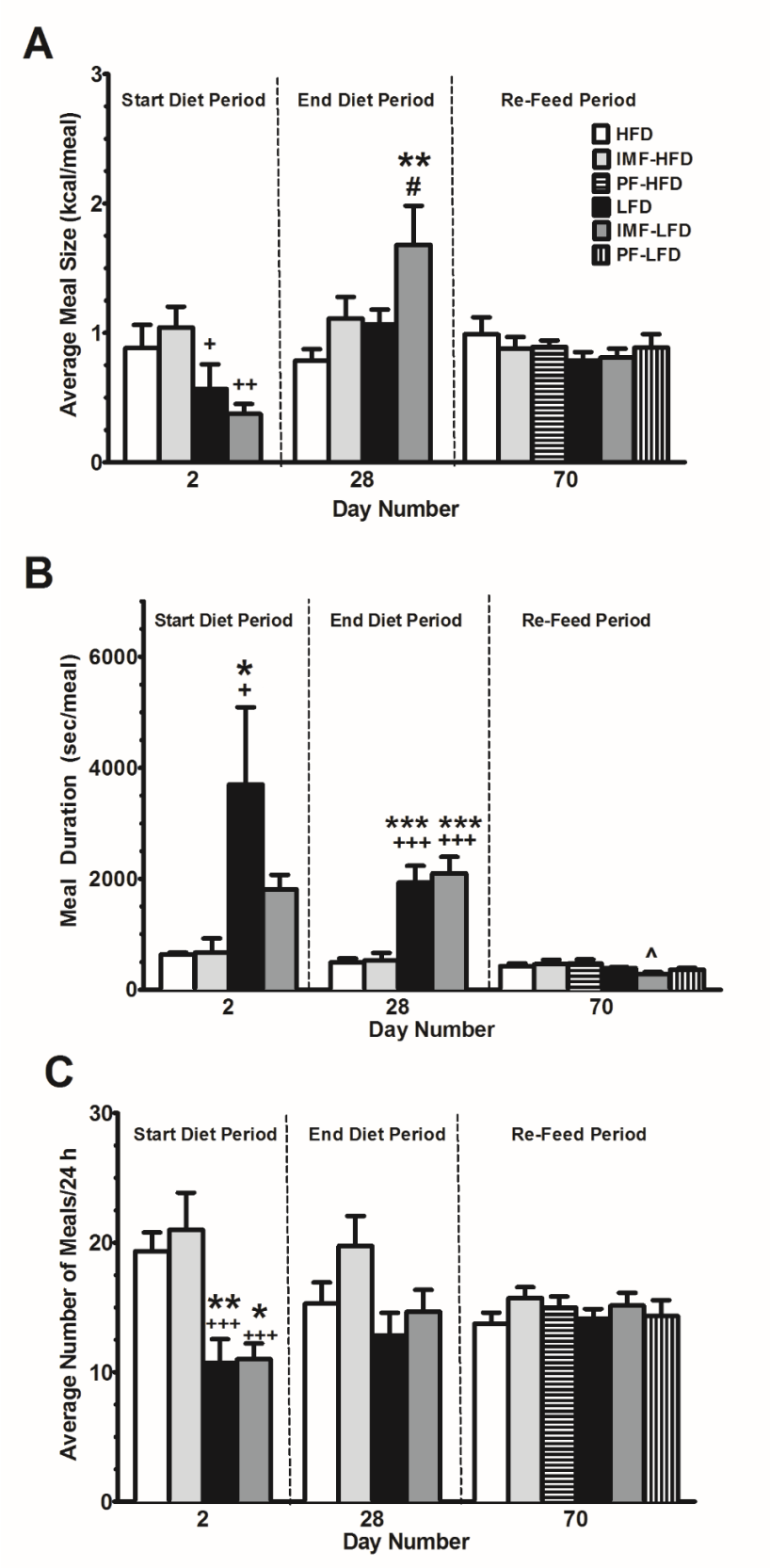
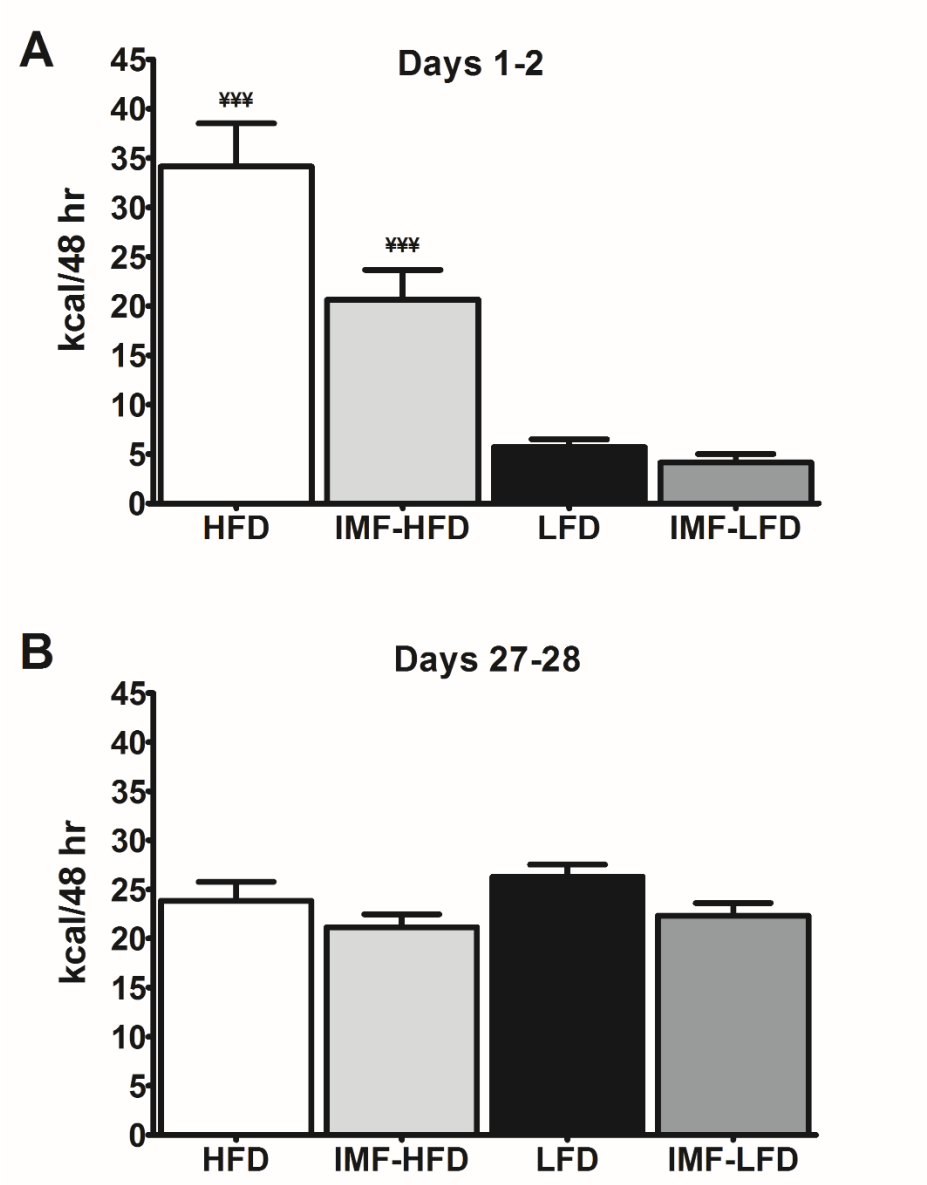

A
Start Diet Period
End Diet Period
Re-Feed Period
HFD
IMF-HFD
PF-HFD
LFD
IMF-LFD
PF-LFD
Average Meal Size (kcal/meal)
Day Number
B
Meal Duration (sec/meal)
Day Number
C
Average Number of Meals/24 h
Day Number

Figure 6.


A
Days 1-2
kcal/48 hr
45
40
35
30
25
20
15
10
5
0
¥¥¥
¥¥¥
HFD
IMF-HFD
LFD
IMF-LFD
B
Days 27-28
kcal/48 hr
45
40
35
30
25
20
15
10
5
0
HFD
IMF-HFD
LFD
IMF-LFD

CHAPTER 5:

Summary and discussion

5. Summary and discussion

Intermittent fasting has recently become a popular alternative weight loss strategy to daily caloric restriction. Most clinical examinations of IMF have been performed in overweight or obese populations and report significant reductions in body weight (~5-8% reduction) after 8-12 weeks of dieting [1-18]. In clinical populations, feelings of hunger experienced during the fasting period are often an obstacle to long-term adherence. Therefore, a small meal of approximately 500 kcal may be incorporated into fasting days (i.e. modified IMF) to improve dietary adherence [19]. A better understanding of the neural and metabolic changes by which IMF promotes weight loss in obesity would provide valuable data for making research-based alterations to IMF protocols to improve long-term compliance. Therefore, the objective of this dissertation was to determine the metabolic, neural, and behavioral outcomes of an alternate day intermittent fasting intervention in diet-induced obese male mice. In each specific aim, a DIO phenotype was produced by placing mice on a 45% high fat diet for 8 weeks followed by an experimental diet period of 4 weeks in which mice continued on *ad libitum* HFD or were switched to IMF-HFD, LFD, or IMF-LFD. In chapter 4, there were additional groups pair fed to IMF-HFD and IMF-LFD and all mice were returned to *ad libitum* HFD for approximately 6 weeks following the diet period. The choice of returning to a HFD, rather than a LFD or chow, allowed for direct comparisons to the HFD control, which remained on this diet for the entirety of the 18-week study. Moreover, use of a HFD provided valuable information on weight regain trajectories following an IMF or caloric restriction diet, which had not been previously investigated. IMF, regardless of dietary fat content,

reproducibly generated significant weight loss by the end of the 4-week diet period (IMF-HFD: ~13-27% reduction; IMF-LFD: ~18-32% reduction).

In addition to gross body weight changes, in chapters 2 and 4 we looked at differences in both fat and lean mass. At the end of the diet period, IMF-HFD, LFD, and IMF-LFD had significantly lower body fat mass than HFD animals and IMF-LFD had greater lean mass than all other groups. Concurrently, IMF and LFD animals also had lower concentrations of plasma leptin. After resumption of an AL HFD for 6 weeks, all differences in both fat and lean mass are washed out. One interesting finding, however, is that pair-fed animals (PF-HFD and PF-LFD) had lower fat mass at the end of the HFD re-feeding period than all other groups. Though we did not record such values, it would be useful to know if PF animals had lower total fat mass at the start of the re-feeding period. It is unclear why IMF-LFD animals retained higher lean mass after the diet period and PF animals lower fat mass after the HFD refeeding period. Others have also reported changes in body composition such as increases in heart, gastrocnemius, tibialis, and diaphragm muscle mass as a percentage of total body weight in IMF animals compared to AL ones fed a chow diet [20]. Chausse et al. found decreases in soleus and plantaris muscle weights, in addition to decreases in epididymal and visceral fat depots, in IMF animals even when normalized for body weight [21].

In addition to body weight and composition, metabolism was assessed through oral glucose and insulin tolerance tests performed at the end of the diet period (chapter 2) and HFD refeeding period (chapter 4). IMF-LFD animals exhibited lower blood glucose over time at the end of the diet period. Though in a different cohort, this effect appears

to be lost after 6 weeks of HFD refeeding. Moreover, PF-HFD and PF-LFD animals demonstrated lower OGTT AUC compared to all other groups at the end of the HFD-refeeding period. One explanation for these differences is perhaps due to greater retained lean mass in IMF-LFD animals and lower fat mass in PF animals. Likewise, IMF-HFD and IMF-LFD mice demonstrated insulin tolerances significantly different from their AL fed counterparts at the end of the diet period and all three dietary groups exhibited lower fasting plasma insulin levels. However, there were no differences between any of the groups at the end of the HFD refeeding period. Though findings during the diet period seem promising, rodent models do not always accurately reflect alterations that occur in human populations. For example, a recent clinical trial in obese adult men and women implemented 8 weeks of IMF followed by 24 weeks of unsupervised dietary intake [17]. Subjects on an IMF diet had significantly lower fasting glucose, but not insulin, levels compared to baseline after the dieting period, but returned to baseline after 24 weeks of unsupervised intake [17].

Another major finding of this study was the fasting specific changes in hypothalamic monoamine contents and hypothalamic gene expression. IMF-HFD and IMF-LFD had higher anterior medial hypothalamic (inclusive of the PVN) NE concentrations and IMF-LFD had higher posterior medial hypothalamic (inclusive of the ARC and VMH) NE concentrations than AL fed counterparts. Moreover, IMF animals had higher relative NPY gene expression in the ARC than HFD and LFD mice. A1 and A2 projections from the caudal hindbrain are a major source of NE in the hypothalamus and are highly involved in the glucoprivic feeding response [22-24]. When these neurons are

lesioned, glucoprivic feeding from 2-DG is completely abolished as is 2-DG induced elevations in NPY and AGRP gene expression in the ARC [23, 25, 26]. We therefore hypothesized that the repeated bouts of glucoprivation experienced during IMF promote enhanced NE signaling to the hypothalamus, subsequently increasing NPY expression and food intake (chapter 3). We approached this question by challenging NPY-GFP tagged animals with an i.p. injection of 2-DG and measuring neural activation in the ARC and PVN, but did not observe any differences between groups in either region. One plausible explanation is that hindbrain signaling, rather than hypothalamic NPY signaling, is primarily responsible for the fasting-specific signaling differences reported in chapter 2. Double labeling for c-Fos and dopamine-β-hydroxylase or tyrosine hydroxylase, common markers for NE and epinephrine containing neurons, in the NTS would likely have yielded greater differences if this were the case.

There were other notable relative gene expression differences as well. *Pomc* expression was lower in IMF-HFD, LFD, and IMF-LFD animals and, though not statistically significant, *Agrp* expression followed the same pattern as *Npy* with a slight elevation in the IMF groups. One intriguing finding was that there were fasting specific increases in ARC *Ghsr* expression, but no differences in plasma ghrelin levels between groups. Clinically, 10-weeks of a modified IMF diet in obese subjects increases plasma ghrelin response to a meal challenge over 120 min, though ghrelin response was not associated with perceived levels of hunger in participants [13].

These fasting specific neural changes are particularly of note as one major obstacle for the long term implementation of IMF is increased feelings of hunger on fasting days

[19]. Moreover, one major criticism of this dietary strategy is prolonged periods of fasting and refeeding will result in disordered eating behaviors [12]. As such, we investigated changes in meal pattern microstructure associated with IMF in DIO animals (chapter 4). Concurrent with body fat mass, cumulative caloric intake was not statistically different between IMF-HFD, LFD, and IMF-LFD at the end of the diet period and all were significantly less than the HFD group. However, despite similar total consumption, meal patterns varied between these groups with LFD and IMF-LFD groups eating significantly longer meals and IMF-LFD eating more kcal/meal by the end of the diet period. Additionally, IMF animals consumed significantly more kcal and at a faster rate than Al fed counterparts in the first meal of the feeding day. After 6 weeks of AL HFD refeeding, all groups demonstrated similar meal patterns regardless of previous diet exposure, suggesting no residual effects of IMF of eating behavior once AL feeding has been restored. One possible explanation for the transient effects of IMF on feeding behavior is that NPY signaling is restored to baseline once the IMF period ends. Though we did not look at NPY expression following the HFD re-feeding period, others have shown refeeding following restriction reduces NPY expression to those of *ad libitum* fed animals [27, 28]. Moreover, it should be noted that the 24-hr caloric deprivation period used in this model is not equivalent to a 24-hour period experienced in humans. Rodents have significantly faster basal metabolic rates and shorter lifespans, indicating that the same period as experienced by a rodent is physiologically much longer than a human [29]. However, the exact period of caloric deprivation time that would be equivalent is difficult to achieve and would be, at best, an approximate estimation.

One limitation of this doctoral work is that only male mice were used for the studies described. There are known sex-dependent differences in food intake and weight loss [30]. Estrogen is protective against fat gain and others have demonstrated that female rats on a high fat, high glucose diet do not gain more weight than chow fed counterparts [30]. Moreover, this same study found that male rats, but not female rats, had significantly lower plasma cholesterol and leptin, but higher adiponectin, levels compared to controls [30]. Additionally, females are susceptible to reproductive complications due to caloric deprivation and weight loss and, therefore, should be considered in future investigations of IMF. Indeed, it would also be of interest to determine sex-specific macronutrient preferences in IMF. While we did not observe any differences in meal patterns after the HFD refeeding period, models of binge-like eating use discreet bouts of access to highly-palatable diet. It is possible that binge-like behavior would be more apparent with an experimental design more closely representing binge models. Intriguingly, there is some preclinical data to suggest that IMF produces anti-depressant and cognitive benefits, though these studies were performed in non-obese, adolescent animals [31, 32]. It would be useful to understand if these beneficial changes remain in a model of weight loss or in older animals.

Taken together, there is great need to develop effective weight-loss and weight-loss maintenance strategies in overweight and obese populations. While pharmacotherapy and bariatric surgical options may help achieve meaningful reductions in body weight, they are often accompanied by significant side effects, high costs, negative perceptions, and even mortality risk. Lifestyle modification through daily CR

provides the most cost effective and comprehensive approach to weight loss, but may be accompanied by feelings of restriction. IMF is a safe, alternative approach to daily CR that may help alleviate such feelings. Nonetheless, it is largely unknown by which mechanisms IMF is driving weight loss and if they differ from CR. This doctoral work provides preliminary data in a clinically relevant model of obesity and suggests fasting-specific alterations in hypothalamic signaling may be contributing to weight loss. A better understanding of these changes in obese animal models is needed to determine the mechanisms driving these alterations and if they are sustainable long-term.

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