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

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Abstract

Alternate day, intermittent fasting (IMF) can be an effective weight loss strategy. However, the effects of IMF on eating behaviors are not well characterized. We investigated the acute 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 to induce diet-induced obesity (DIO), mice were either continued on *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. By the end of the diet period, all 5 groups had lost weight compared with HFD group, but after 6 weeks of HFD re-feeding all groups had similar body weights. On (Day 2) of the diet period, IMF-HFD had greater first meal size and faster eating rate compared with HFD. Also, 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 HFD re-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 are only transiently altered during alternate day intermittent fasting for weight loss in obese male mice.

Key Words: Dieting, meal patterns, intermittent fasting, caloric restriction, feeding, glucose homeostasis, body fat

1. 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. 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 duration of 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 a weight loss of 3-8% in both overweight and obese adult populations [6-13].

Prolonged periods of reduced calorie intake have been reported in individuals with eating disorders [14, 15]. Indeed, past prolonged bouts of CR have been associated with the 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, is 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 (DIO) and leptin deficiency, are associated with larger meal sizes [29-31]. Not only is daily caloric intake dependent on meal size, but the number of

meals and interaction between meal size and number are 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 effects of these type of dietary approaches.

The goal of the present study was to evaluate meal patterns as a consequence of IMF in 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 to determine how this type of dietary strategy influences eating behaviors.

2. Materials & Methods

2.1. 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 (HFD; 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 (LFD; 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.

2.2. 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 sec and a minimum of 0.02 g consumed, as recommended by Research Diets. Meal patterns were measured over the 48 hours of the experimental diets (Days 1 & 2). That is, meal patterns were measured in IMF groups for the 24-h food deprivation followed by 24 h re-feeding. For the LFD groups, this was the first 48 h of diet switch. This was repeated for the last 48 h (Day 27 & 28) of the diet period, see Figure 1. Values are reported for the second 24 h 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/min), and duration (sec) 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 h recording periods were evaluated.

2.3. 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 HFD 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 beginning 5 h 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 min post-gavage. The test was ended at 180 min, 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 (ITT) was performed after a 5 h 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 at 15, 30, 60, 90, and 120 minutes post-injection. Area under the curve (AUC) for blood glucose was calculated by the linear trapezoidal method for both OGTT and ITT.

2.4. 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. Fisher's Least Significant Difference post-hoc tests were 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 $\alpha = 0.05$.

3. Results

3.1. 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 $18.35 \pm 2.63\%$ calorie restriction compared to *ad libitum* fed HFD mice. Likewise, IMF-LFD mice received a $29.31 \pm 5.91\%$ calorie restriction compared to *ad libitum* fed HFD mice. The equivalent amount of diet was received by the respective PF groups. At the end of the 4-week diet period 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 weight 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.

3.2. 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 groups on an 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 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 min 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.

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

The first meals 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.

3.3.1. 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).

3.3.2. 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).

3.3.3. 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 ($F(1, 24) = 14.0, P < 0.005$), and 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).

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

Average meal size, duration, and number of meals were recorded during 24-h 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).

3.4.1 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 meals 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.

3.4.2. 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.

3.4.3. 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 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.

3.5. 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.

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 diet (45% fat) diet for 8 weeks. DIO mice were placed on 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 as well as an 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 on which IMF-LFD group was 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 that of the IMF-HFD and HFD groups.

By the end of the diet period, all groups had reduced body weight compared with HFD controls. 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 likely a 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 caloric 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 a high-fat diet for 6 weeks and restored to a similar body weight as the HFD group by 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 slower meal duration did not have an overall influence on the meal patterns since there were not any significant differences in other meal parameters at Day 70 for the IMF-LFD. 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 an 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 the 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 groups compared with *ad libitum* fed groups. In particular, we previously observed that the IMF groups had increased RER ($\text{v.CO}_2/\text{v.O}_2$), 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 a 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 increase 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) of 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 with the *ad libitum* fed HFD group over the diet period. One major limitation of our use of 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 ≥ 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 previously exposed PF groups had lower fat mass after 6 weeks on the HFD. Even though there were no differences in body weight between the groups on day 78, after 49 days on re-feeding of HFD, one possibility is that the PF groups had a different trajectory of restoration of body composition 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 previously 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 with the lean chow-fed groups after weight regain, an effect that was not observed in the continuously restricted group [27]. One notable difference between Seimon and colleagues study and the present study is that we exposed the mice to a HFD following the diet period. Nonetheless, the Seimon and colleagues study illustrates how body fat can be differentially affected 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 meal patterns during the diet period. An expected sex-dependent difference would likely influence the time to weight loss and recovery 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 that 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.

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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. During 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 $\pm$ SEM. **A:** Bodyweights (g) for all groups during the diet period. **B:** Body weights during re-feeding for all groups previously exposed to 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 $\pm$ 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, and 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 $\pm$ 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 $\pm$ 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 h 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 $\pm$ SEM. **A:** Days 1-2, and **B:** Days 27-28. ¥¥¥ indicates difference from all other groups ($P < 0.001$).

Fig. 1

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