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Complete List of Authors:	Delahaye, Laura B.; University of Memphis, School of Health Studies Bloomer, Richard J.; University of Memphis , School of Health Studies Butawan, Matthew B.; University of Memphis, School of Health Studies Wyman, Jacqueline M.; University of Memphis, School of Health Studies Hill, Jessica; University of Memphis, School of Health Studies Lee, Harold W.; University of Memphis, School of Health Studies Liu, Andrew C.; University of Memphis, Department of Biological Sciences McAllan, Liam; University of Tennessee Health Science Center, Department of Pediatrics Han, Joan C.; University of Tennessee Health Science Center, Department of Pediatrics; University of Tennessee Health Science Center, Department of Physiology; Le Bonheur Children's Hospital, Children's Foundation Research Institute van der Merwe, Marie; University of Memphis, School of Health Studies
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Time-restricted feeding of a high fat diet in C57BL/6 male mice reduces adiposity, but does not protect against increased systemic inflammation.

Laura B. Delahaye[1], Richard J. Bloomer[1], Matthew B. Butawan[1], Jacqueline M. Wyman[1], Jessica L. Hill[1], Harold W. Lee[1], Andrew C. Liu[2], Liam McAllan[3], Joan C. Han,[3,4,5] and Mariè Van der Merwe[1*]

[1]School of Health Studies, [2]Department of Biological Sciences, University of Memphis, TN; [3]Department of Pediatrics and [4]Department of Physiology, University of Tennessee Health Science Center, Memphis, TN; [5]Children's Foundation Research Institute, Le Bonheur Children's Hospital, Memphis TN

***Corresponding author:**
Mariè van der Merwe, PhD
School of Health Studies
310 Elma Roane Fieldhouse
University of Memphis
Memphis, TN, 38152
Phone: 901-678-3476
Fax: 901-678-3591
Email: mvndrmrw@memphis.edu

Abstract

Time-restricted feeding (TRF) limits duration of food availability without altering diet composition and can combat obesity in humans and mice. For this study we evaluated the effect of timing of food access during a TRF protocol on weight gain, adiposity and inflammation. Young C57BL/6 male mice were placed on a 45% high fat (HF) diet for 8 weeks. Food access was unrestricted (HF) or restricted to 6 hours per day, either for the first half (HF-early) or the second half (HF-late) of the active phase to resemble a window of time for food consumption early *vs* late in the day in a human population. Weight, obesity-associated parameters and inflammation were measured. TRF reduced weight gain over the 8-week period when consuming the same high fat diet. Consistent with decreased weight gain in the TRF groups, body fat percentage, liver triglyceride, plasma leptin and cholesterol levels were reduced. Adipose tissue inflammation, measured by $CD11b^{+}F4/80^{+}$ macrophages infiltration, was reduced in both TRF groups, but systemic TNF-α were increased in all groups consuming the high fat diet. The HF-late group gained more weight than the HF-early group and had increased insulin resistance, while the HF-early group was protected. Therefore, a TRF dietary protocol is beneficial for weight management when consuming a high fat diet, with food consumption earlier in the day showing greater health benefits. However, increased inflammatory markers in the TRF groups suggest that diet components can still increase inflammation even in the absence of overt obesity.

Keywords: obesity, circadian rhythm, intermittent fasting, time-restricted feeding, inflammation

Introduction

The increase in obesity and metabolic syndrome have fueled the search for dietary approaches to prevent the accumulation of excess weight. Long-term caloric restriction, with adequate intake of nutrients, can reduce body weight and obesity-associated diseases, including type 2 diabetes, hypertension and cardiovascular disease (Papadaki et al. 2013), but this highly successful approach is challenging to maintain over an extended period of time (Hemmingsson et al. 2012). Intermittent fasting (IF) is an increasingly popular dietary approach for weight loss and metabolic health. IF improves blood lipids, glycemic control, reduces circulating insulin, decreases blood pressure, and reduces fat mass (Gotthardt et al. 2015; Anton et al. 2017; Arnason et al. 2017; Patterson et al. 2017). One particular form of IF, time-restricted feeding (TRF), limits the time and duration of food availability with no planned change in caloric intake. This type of feeding regimen allows individuals *ad libitum* energy intake of their normal diet within a set window of time (e.g., 3-4 h, 7-9 h or 10-12 h), which leads to an extended fasting period each day (Rothschild et al. 2014). This specific form of IF has been shown to heighten parasympathetic activity in the autonomic neurons that innervate the gut, heart and arteries improving gut motility and decreasing heart rate and blood pressure (Cherif et al. 2016). In addition, restriction of food availability to a window of time each day results in a reorganization of physiology and behavior directed by food-entrainable oscillators associated with the circadian clock (Stephan 2002). Coordinated responses of the clock genes in various organs are important for energy use and storage and loss of synchronized activity between the central and peripheral clocks can manifest in metabolic syndrome (Chung et al. 2011; Kino and Chrousos 2011; Cho et al. 2012; Hatori et al. 2012; Neufeld-Cohen et al. 2016).

Both human and mouse studies demonstrate that TRF dietary protocols have metabolic implications that protect against obesity and the development of metabolic syndrome (Manoogian and Panda 2017; Patterson and Sears 2017). Controlled mouse studies demonstrate that consuming a high fat diet in a time-restricted manner protected against excessive body weight gain and adipose tissue-associated inflammation, and retained the cyclical expression of circadian oscillators. Moreover, TRF can also stabilize and reverse the progression of metabolic diseases in animals with preexisting obesity and type 2 diabetes (Chaix et al. 2014; Chung et al. 2016).

The objective of this study was to determine if the protective role of TRF is maintained, when high fat (HF) feeding occurs at different time points during the day. More specifically, we determined if there are differences in metabolic outcomes and immune cell responsiveness when high fat feeding is restricted to either the beginning or end of the 12-hour active phase. These feeding periods represent a window of food access during the first half (breakfast-lunch) or the second half (lunch-dinner) of the day when translating to a typical human eating schedule. We show that TRF protects against the development of obesity, increased liver triglycerides and adipose-tissue associated inflammation, but not against increased systemic tumor necrosis factor-α (TNF-α) when consuming a high fat diet. Our data further demonstrate that the group with access to food during the first half of the day gained less weight per kilocalorie (kcal) than the group eating later.

Methods and Methods

All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental animals and were approved by the University of Memphis Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and reduce the number of animals used.

Experimental Animals

Nine week old C57BL/6 male mice were purchased from Envigo Laboratories Inc. (Pratville, AL) and housed in a USDA approved animal facility with regulated light-dark cycle of 24 h at room temperature (22 ± 2 °C). Male mice were used in this study as they tend to gain weight faster than females and would allow for a bigger effect size during the 8 week study (Yang et al. 2014). 2-3 animals were housed per cage and mice were entrained to a reversed light-dark schedule (12h light: 12h dark), with lights off between the hours of 7:00-19:00 for 3 weeks (Figure 1). This timeframe has previously been shown to be sufficient for entrainment (Cao et al. 2013; Cao et al. 2015). The reversal of the light cycle was done so that food change time (7:00 and 13:00) coincided with working hours. During entrainment, all mice had *ad libitum* access to water and unpurified standard rodent chow (Teklad global 2018, Envigo Laboratories Inc.; 18% fat, 58% carbohydrates, 24% protein; 3.1 kcal/g). Upon completion of the entrainment period, mice were randomly assigned into one of four diet groups (n = 10-11 mice per group). Group 1 (Chow) continued *ad libitum* feeding of the standard rodent chow. This diet was continued, as it supports normal growth without inducing obesity and metabolic dysfunction. The remaining three groups were all fed the same HF diet (D12451, Research diets, Inc., New Brunswick, NJ; 45% fat (predominantly lard), 35% carbohydrate and 20% protein; 4.73 kcal/g). This diet is used to generate diet-induced obesity models (Van Heek et al. 1997; Akbay et al. 2004; Zeng et al.

2013; Hennigar et al. 2015; Cui et al. 2016). Group 2 (HF, control group) were fed *ad libitum*, while groups 3 and 4 had time-restricted access to food for only 6 hours/day. Group 3 (HF-early) had access to food at the beginning of the active phase (7:00 - 13:00), while group 4 (HF-late) had access during the second half of the active phase. (13:00 - 19:00) All animals had *ad libitum* access to water. The macronutrient composition of the HF diet is described in Table 1. All mice were fed their respective diets and maintained on assigned feeding schedules for 8 weeks. Food intake was monitored daily and body weight measured twice a week. Body composition was determined for a subgroup of the animals one day prior to sacrifice using an Echo MRI 1100 (EchoMRI® LLC, Houston, TX). To avoid circadian rhythm induced differences, all mice were sacrificed at the same time (7:00) using CO_2 inhalation. This resulted in differences in fasting times for different groups. The chow and HF groups were fasted for 10 hours (21:00 – 7:00) prior to sacrifice, while animals on the TRF protocols remained on their respective fasting schedules. At sacrifice, HF-early would have been fasted for 18 hours, while HF-late would have been fasted for 12 hours. Blood was collected immediately prior to sacrifice via the facial vein. Spleen, liver, heart and epididymal white adipose tissue (eWAT) were harvested and immediately processed or stored at -80 °C.

Glucose Tolerance Test and HOMA-IR

One week prior to sacrifice, mice were subjected to an intraperitoneal glucose tolerance test (IPGTT). As insulin secretion is under circadian control, the IPGTTs were performed at the same time (5:00) for all animals. The chow and HF groups were fasted for 7 hours (22:00 – 5:00), while animals on the TRF protocol remained on their respective fasting schedule. The HF-early group fasted for 16 hours and HF-late for 10 hours at the time of IPGTT. Baseline fasting blood

glucose levels were measured from the tail vein using a glucometer (Onetouch Ultra® 2 Meter, Bayer Healthcare, Tarrytown, New York). Glucose (1g/kg of body weight) was administered by intraperitoneal injection and blood glucose levels were measured every 30 minutes for 90 minutes via the tail vein. In addition, fasting glucose (TR15103/1530-500, Thermo Electron. Louisville, CO) and insulin (Mouse Ultrasensitive Insulin ELISA, Alpco, Salem, NH) were measured in plasma collected at sacrifice and used to estimate the index of insulin resistance. The formula for the homeostatic model of insulin resistance (HOMA-IR) was used for this estimation (fasting insulin [mU/L] x fasting glucose [mmol/L])/22.5) (Seimon et al. 2016).

Blood parameters

Blood was collected into EDTA-treated tubes at sacrifice. Cells were pelleted by centrifugation at 2000 x g for 15 minutes at 4°C and plasma removed and stored at -80°C. Levels of leptin, TNF-α and Interleukin 6 (IL-6) were measured using a magnetic bead assay (LXSAMSM, R&D Systems, Minneapolis, MN) and analyzed on the Luminex® MAGPIX® platform with xPONENT® software to distinguish bead color and fluorescence intensity.

Cholesterol (TR13421/2350-250, Thermo Electron. Louisville, CO) and triacylglycerol (TR22421/2780-250, Thermo Electron. Louisville, CO) levels were measured following standard enzymatic procedures as described by the reagent manufacturer.

Tissue Collection and Histology

Immediately after sacrifice, the liver and epididymal adipose tissue were harvested and weighed. A representative sample of each tissue was fixed in 10% neutral buffered formalin solution (Fisher Scientific Co. LLC) for 48-72h. Samples were then dehydrated in a series of graded

ethanol solutions, cleared with xylene embedded in paraffin, and cut into 4 μm sections and stained with hematoxylin and eosin (H&E). Histological analysis and documentation were performed using an Imager M2 microscope (Axiocam MRC, Zeiss, Oberkochen, Germany). The size of individual adipocytes was determined using Axiovision r4.8.2 software. Two to three fields were quantified per mouse, with 10-11 mice per group, generating 300-400 individual data points per group.

Liver triglycerides

Liver triglyceride concentration was measured using a commercially available kit (10010303, Cayman chemical, Ann Arbor, MI) and performed according to the manufacturer's instructions.

Immune cell isolation, flow cytometry and in vitro proliferation.

The spleen and remaining adipose tissue were immediately processed for cell isolation and immune population analysis. Splenocytes were isolated in RPMI 1640 solution containing 2% FBS by pressing the spleen through a sterile 40 μm nylon cell strainer using a syringe plunger to generate a single cell suspension. Red blood cells were lysed using ACK Lysing buffer (A10492-01, Gibco, Gaithersburg, MD) and cells were washed with 1XPBS containing 2% FBS.

Epididymal adipose tissue was cut into small pieces and placed in DMEM (SH3024301, HyClone, GE Healthcare Life Sciences, Logan, UT; 0.6g/2mL), containing 2 mg/mL type II collagenase (Worthington, Lakewood, NJ), and incubated at 37˚C with continuous agitation (240 rpm) for 40 minutes. After incubation, samples were diluted with DMEM and filtered through a

40 µm nylon sterile cell strainer, centrifuged at 500 x g for 10 minutes at 4°C and pellets re-suspended in 1xPBS with 2% FBS (Lynch et al. 2012).

Prior to antibody staining, all cells were incubated with Fc block (FcX, Biolegend, San Diego, CA) at room temperature for 10 minutes. LIVE/DEAD™ Fixable Aqua (Life Technologies, Eugene, OR) was used to exclude dead cells. The following fluorescently conjugated antibodies were used for cell identification: PE anti-TCRαβ (clone H57-597, Biolegend), APC-Cy7 anti-B220 (clone RA3-6B2, Biolegend), FITC anti-CD11b (clone M1/70, Biolegend) pacific blue F4/80 (clone BM8, Biolegend). All samples were fixed using Fixation/Permeabilization solution (eBioscience, San Diego, CA) overnight at 4˚C according to the manufacturer's instructions. For the *in vitro* proliferation assay, single cell suspensions were prepared aseptically from the spleens as previously described (Pillai et al. 2007). Total splenocytes were labeled with Cell Proliferation Dye eFluor™ 450 (eBioscience) according to the manufacturer's instructions. Cells were cultured at a concentration of 1x10^6 cells/well in a 96 well U-bottom plate in RPMI 1640 supplemented 10% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 nM) and 2-ME (55 mM). Cells were stimulated with 10 µg/mL *Escherichia coli* lipopolysaccharide (LPS) for 72 h in 5% CO_2 at 37°C. At 72 h, splenocytes were labeled with PE-Cy7 anti-CD3 (clone 145-2C11, Biolegend), APC-Cy anti-B220 (RA3-6B2, Biolegend) and LIVE/DEAD™ Fixable Aqua. Cells were fixed overnight at 4°C using Fixation/Permeabilization solution (eBioscience, San Diego, California).

All samples were analyzed using a LSR II Flow Cytometer (BD Biosciences, San Jose, California) and BD FACSDIVA software (BD Biosciences, San Jose, CA). The data obtained were further analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

Statistical Methods

The D'Agostino & Pearson test was used to test for the normality of the data. All data are presented as means ± SEM and statistical significance was established at $P < 0.05$. One-way analysis of variance (ANOVA) and post-test Tukey were used to compare group means. For weight and glucose tolerance data a two-way ANOVA with Tukey's multiple comparison test was performed. All statistical analysis was performed with Graphpad Prism Software (Version 7, San Diego, CA)

Results

Time-restricted high fat feeding protected against weight gain.

To determine the effect of TRF on weight gain, we evaluated body weight, food intake and feed efficiency for all groups (Chow, HF, HF-early and HF-late) (Figure 2). The HF control group gained significantly more weight over the 8-week period than all other groups (HF 16.43 ± 1.86 g, Chow 5.34 ± 1.9 g, HF-early 5.56 ± 2.59 g, HF-late 8.88 ± 1.94 g; $P < 0.001$) (Figure 2A-B). The HF group did consume approximately 15% more calories ($P < 0.05$) with no difference in consumption between the Chow, HF-early or HF-late (Figure 2C). HF-late group also gained significantly more weight than either the Chow or the HF-early mice ($P < 0.01$) (Figure 2B), despite no difference in calorie intake. Energy efficiency calculated as weight gained (mg) per

kcal consumed was significantly higher for the HF group *vs.* Chow, HF-early or HF-late ($P < 0.0001$), with HF-late also higher compared to Chow and HF-early ($P < 0.01$) (Figure 2D).

Time-restricted feeding decreased adiposity and adipose associated parameters.

Body composition and eWAT were evaluated for obesity related changes. Fat free mass was similar between Chow, HF and HF-late (22.52 ± 0.88 g, 23.23 ± 0.96 g, HF-late 22.23 ± 1.33 g), with HF-early (21.19 ± 0.78 g) being slightly reduced relative to HF ($P = 0.01$) (Figure 3A). Total fat mass and eWAT weight were significantly higher for the HF group than for all other groups ($P < 0.01$), with no significant differences between the two time-restricted groups (HF-early *vs.* HF-late; body fat mass, $P = 0.87$; eWAT weight, $P = 0.36$) (Figure 3B, C). Consistent with increased adiposity, the adipokine leptin was increased in plasma of the HF group ($P \leq 0.01$) with no difference between HF-early and HF-late groups (Figure 3D). Interestingly, the HF-late group, but not HF-early group was significantly different from the Chow group (HF-late *vs* Chow $P \leq 0.02$) for eWAT weight and Leptin (Figure 3C, D). To determine whether the increase in overall adiposity correlated with adipocyte size, we evaluated hematoxylin and eosin stained epididymal adipose samples. Average adipocyte size was increased for all groups consuming the high fat diet, irrespective of feeding schedule (HF *vs.* Chow, $P = 0.0003$; HF-early or HF-late *vs*. Chow, $P = 0.01$) (Figure 3E). Interestingly, there was no difference between *ad libitum* HF vs the TRF groups (Figure 3E, HF vs HF-early, P = 0.81; HF vs HF-late, P = 0.42).

Immune cell infiltration into adipose tissue is an indicator of cell stress and adipose tissue-associated inflammation. Immune cells were isolated from 0.6 g of eWAT and analyzed by flow

cytometry. $CD11b^{+}F4/80^{+}$ macrophage numbers were higher in the HF group than in all other groups ($P < 0.0001$) with no difference between Chow, HF-early or HF-late groups ($P = 0.9$, Figure 3F).

While there was no difference in plasma triacylglycerol levels between any of the groups, cholesterol was increased 30-40% in the HF group *vs.* all other groups ($P < 0.0001$) (Table 1).

Time-restricted high fat feeding alters glucose homeostasis.

Obesity is highly associated with altered glucose homeostasis and diabetes. An IPGTT was performed one week prior to sacrifice after a fasting period to determine the effect of TRF on glucose clearance. Due to fasting schedules of time-restricted feeding groups, the HF-early and HF-late fasted for longer periods than the HF and Chow groups. Also, fasting occurred overnight which correlates with the light/inactive phase. Plasma glucose increased for all groups to a maximum 30 minutes after glucose administration. The time-course of glucose clearance was delayed in the HF group and glucose remained elevated at 90 minutes (Figure 4A, *left panel*). The AUC (Area under Curve, Figure 4A, *right panel*) was greater for HF compared to all groups ($P < 0.001$), with no difference among Chow, HF-early or HF-late groups. Fasting glucose and insulin levels were also measured in plasma collected from the facial vein at sacrifice. Glucose and insulin were higher in HF and HF-late groups *vs* the Chow and HF-early groups (Table 1) corresponding to a higher HOMA-IR (HF *vs*. Chow, $P < 0.01$; HF *vs*. HF-early, $P < 0.0001$; HF-late *vs* HF-early, $P = 0.0001$). There was no difference in HOMA-IR between HF and HF-late (Figure 4B).

Time-restricted feeding protects from triglyceride accumulation in the liver.

Liver weight was significantly increased in the HF group (1.59 g ± 0.36), compared to Chow (1.125 g ± 0.13), HF-early (0.93 g ± 0.1) or HF-late (1.14 g ± 0.09) *(P* < 0.0001) (Figure 5A). It is known that hepatic steatosis occurs when hepatic fatty acid uptake from plasma and *de novo* fatty acid synthesis are greater than fatty acid oxidation and export (Fabbrini et al. 2010). To determine the extent of fatty liver development in the different groups, liver triglyceride concentration was measured and hematoxylin and eosin stained liver samples were examined for fatty deposits. Although histological examination suggest some fat accumulation (Figure 5C), triglyceride concentration was significantly increased only in the HF group (HF *vs* Chow, HF-early or HF-late, $P < 0.01$).

Systemic inflammatory molecules are not reduced by time-restricted feeding of a high fat diet.

Increased inflammation is associated with obesity and its metabolic dysfunction. To test whether TRF is protective against systemic inflammation we measured plasma IL-6 and TNF-α levels. Despite great variation in the TRF groups, there was no difference in IL-6 levels between groups (Figure 6A). There was a significant increase in TNF-α in all HF diets group *vs* Chow ($P < 0.05$), irrespective of timing of feeding. (Figure 6B).

To further assess the effect of dietary protocol on immune parameters, we measured LPS-induced *in vitro* proliferation of $B220^+$ splenocytes isolated from mice on different dietary protocols. While no proliferation was detected with unstimulated cells in the Chow and TRF groups, there was a small, but non-significant increase in proliferation of $B220^+$ eFluor®450-labeled splenocytes in the HF group (Figure 6C-D). *In vitro* LPS stimulation increased

proliferation of all groups, with the HF group being the most proliferative (HF vs Chow, 85.83 ± 2.8 *vs* 54.75 ± 1.2, $P < 001$). Both time-restricted groups (HF-early and HF-late) had a small, but non-significant increase in proliferation after stimulation (HF-early *vs.* Chow, 67.3 ± 4.7 *vs.* 54.75 ± 1.2; HF-late *vs* Chow, 66.53 ±12.79 *vs* 54.75 ±1.2) (Figure 6C-D).

Discussion

Current TRF dietary protocols do not limit food intake or alter dietary composition, but allow access to food in a time-dependent manner. Several studies indicate that TRF protocols can protect against obesity related metabolic dysfunctions (Hatori et al. 2012; Sherman et al. 2012; Shi et al. 2013; Chaix et al. 2014; Chung et al. 2016). The benefit of TRF protocols are suggested to result from the reestablishment of cyclicity by nutrient-entrained oscillators as perturbation of circadian rhythms associated with *ad libitum* feeding contribute to obesity and diabetes. In addition, high fat diets can alter circadian cycles by dampening the amplitude of clock gene expression, but Hatori et al. (Hatori et al. 2012) demonstrated that a TRF protocol, even when consuming a high fat diet, is protective against obesity and its associated metabolic diseases. By restricting feeding to a small window of time that is aligned with the circadian cycle, and allowing for an extended period of fasting, the coordination between the circadian and metabolic regulators are restored.

To determine the effect of temporally shifting the activation of peripheral oscillators from the activation of the central clock, preclinical rodent studies have examined limiting food access to the light or inactive phase only and thereby completely uncoupling feeding from the central clock. In addition, studies have also examined manipulation of feeding times during the normal

awake/active period, showing that diet composition at different times and timing of feeding effects weight gain and metabolic health (Bray et al. 2010; Wu et al. 2011). The TRF protocol used in the current study also manipulated feeding times during the active period by restricting daily food access for 18 hours and allowing 6 hours of *ad libitum* consumption of a 45% high fat diet. The 6 hours of food access was either at the first half or the second half of the 12 hour active phase.

Results from this study confirm that TRF of a HF diet protects against the increased body weight gain usually associated with *ad libitum* access of this diet. Notably, at the end of the 8-week feeding protocol, mice consuming the high fat diet *ad libitum* gained 2-3 times more weight and had 38-45% more body fat than time-restricted animals fed the same high fat diet. Interestingly, the "late" time-restricted group also gained significantly more weight, albeit not to the level of the *ad libitum* group. This weight gain correlated with increased feed efficiency (as calculated by weight gained per calorie consumed), suggesting that restricting food access towards the end of the active phase increases the potential for weight gain, while timing of food access to earlier in the active phase is more beneficial for weight management. Body composition was similar between the "early" and "late" time-restricted groups, with no significant difference in adipose tissue mass or systemic leptin levels.

Interestingly, glucose clearance as measured by an intraperitoneal glucose tolerance test is maintained by the TRF of the high fat diet, but after 8 weeks on the specific protocols, the HOMA-IR was increased in the group that consumed their meal at the end of their active phase to similar levels as the high fat *ad libitum* group. The levels of insulin and fasting glucose was dramatically reduced in the HF-early group, possibly due to the longer fasting time at sacrifice

due to feeding schedule. Although liver fatty deposits were evident in all animals consuming the high fat diet, an association also seen in humans (Mollard et al. 2014), the TRF groups had decreased liver weight and liver triglyceride concentration, suggesting that TRF protocols can protect against fatty liver development. (Hatori et al. 2012; Adamovich et al. 2014; Chaix et al. 2014; Chung et al. 2016).

Insulin resistance, fatty liver, and low grade systemic inflammation are all hallmarks of obesity (Tilg and Moschen 2008) and the release of TNF-α by macrophages recruited by stressed, hypertrophied adipocytes is a well-established consequence of increased adiposity (Weisberg et al. 2003; van der Heijden et al. 2015). Although adipocyte size was increased in all high fat diet groups, the inflammatory environment within the adipose tissue was increased only in the HF *ad libitum* group as evident by $F4/80^{+}$ macrophages infiltration, not seen in the TRF groups. These findings corroborate previous studies of inflammation and TRF that reported reduced inflammatory molecules in the adipose tissue itself (Sherman et al. 2011; Hatori et al. 2012).

Surprisingly however, systemic TNF-α levels were increased to the same level in all the high fat diet groups, irrespective of feeding schedule. This result suggests that the release of this inflammatory molecule is a consequence of the high fat diet and not only eWAT associated macrophages. Consistent with previous work showing that dietary fatty acid composition and caloric restriction of a high fat diet can alter immune responses (Park et al. 2013; Wang et al. 2013), we demonstrate an increase in *in vitro* $B220^{+}$ splenocyte proliferation in response to LPS when exposed to a high fat diet. Although TRF protocols showed increased responses, it was not to the same extent as the *ad libitum* fed HF group. This result suggests that the composition of

the diet directly or indirectly acts to increase cell responsiveness to an immune stimulus and increased expression of Toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain (NOD) 2 have been observed with the consumption of high fat diets (Xu et al. 2008; Kim et al. 2011; Zhang et al. 2015; Sutter et al. 2016). Additionally, high fat diet-induced changes in intestinal microbiome and gut-permeability might also contribute to the increased activity of immune cells (Kim et al. 2012; Lam et al. 2012; Lam et al. 2015).

Conclusion

TRF protocols can reduce the development of obesity associated with a high fat diet. Timing of food consumption does affect weight gain as the group that was restricted to food access during the second half of the day gained slightly more weight than the group with earlier access. Interestingly, despite the protection of TRF against many obesity-related parameters, this dietary protocol did not protect against increased systemic TNF-α levels. This result suggests that consuming a high fat diet, rich in saturated and omega-6 fatty acids, in a time-restricted manner might be beneficial for weight management, but does not protect against diet-induced systemic inflammation.

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edited manuscript. MV had primary responsibility for final content. All authors read and approved the final manuscript.

The authors have no conflict of interest to report.

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Table I. Composition the diets.

	Unpurified Chow	**High Fat Diet (D12451)**
Macronutrients		
Protein, *% kcal*	24	20
Carbohydrate, *% kcal*	58	35
Fat, *% kcal*	18	45
Total, *% kcal*	100	100
Energy, *% kcal*	3.1	4.7

Table II. Fasting plasma lipid, glucose, and insulin levels after 8 weeks on a TRF protocol.

Parameter	Chow	HF	HF-early	HF-late
Cholesterol (mg/dL)	142.1 ± 19.98	240.3* ± 42.38	142.6 ± 26.09	165.3 ± 15.55
Triacylglycerol (mg/dL)	104.9 ± 40.26	98.08 ± 23.46	97.26 ± 23.54	101.5 ± 16.49
Glucose (mg/dL)	187.1 ± 43.9	263.3† ± 61.98	154.3 ± 43.88	216‡ ± 62.51
Insulin (mU/L)	22.78 ± 14.44	30.09§ ± 9.12	12.25 ± 5.56	29.84§ ± 11.01

*HF vs Chow, HF-early, HF-late (P<0.0001)

†HF *vs* Chow, HF-early (P<0.05)

‡HF-late *vs* HF-early (P<0.05)

§HF, HF-late *vs* HF-early (P<0.01)

FIGURE 1

Schematic outline of the four different dietary protocols followed in this study.

Mice were entrained to a reversed light cycle. The control groups (Chow and HF) were allowed to feed *ad libitum*. The time-restricted feeding groups were allowed to access to the high fat diet for either the first 6 hours or the second 6 hours of their active phase.

FIGURE 2

TRF reduces weight gain and feed efficiency.

C57BL/6 male mice were fed a standard rodent chow or a 45% high fat diet, either *ad libitum* or in a time-restricted manner, for 8 weeks and body weight were monitored (A, B). Total amount of calories consumed (C) and feed efficiency (D) were calculated. Values are mean ± SEM, n= 10-11 mice per group. *The HF group differed ($P < 0.05$) from all other groups for all parameters. #HF-late group differed ($P < 0.05$) from all other groups for weight gain and feed efficiency.

FIGURE 3

TRF reduces adiposity and adipose associated parameters

Body composition (A-B, n = 4-7 per group), eWAT (epididymal white adipose tissue) weight (C) and plasma leptin (D) levels after 8 weeks on various dietary protocols. Values are mean ± SEM, n = 10-11 per group. * The HF group differed ($P < 0.05$) from all other groups. # Chow differed from HF-late ($P < 0.05$) for both eWAT weight and Leptin. (E) Adipocyte cell size measured using light microscopy (2-3 sections were quantified per mouse, n = 6-9 per group). Adipocyte cell size were increased in all groups consuming the HF diet *vs* Chow control *(P <

0.05). (F) Percentage of $CD11b^{+}F4/80^{+}$ macrophages of live cells harvested from 0.6 g of eWAT. HF differed from all groups. ***$P \leq 0.0001$ (n=9-11 per group)

FIGURE 4

TRF with food restricted to early in the active phase improves glucose tolerance and HOMA-IR

Intraperitoneal glucose tolerance test (A, *Left panel*) were performed on all groups following their respective diet/fasting protocols. Area under the glucose curve calculated. (*Right panel*) Values are mean ± SEM, mice = 8-11 per group **HF group differed (AUC, $P < 0.001$) from all other groups. (B) HOMA-IR = fasting glucose (mmol/L) X fasting insulin (mU/L)/22.5. HF differed from Chow (*$P < 0.01$) and HF-early (***$P \leq 0.0001$). HF-early differed from HF-late (***$P = 0.0001$)

FIGURE 5

TRF protects against liver triglyceride accumulation

Liver weight (A, ***HF group differed from all other groups, $P < 0.0001$) and liver triglyceride concentration (B, *HF group differed ($P < 0.01$) from all other groups). Values are mean ± SEM, mice = 10-11 per group. (C) Representative hematoxylin and eosin (H&E) - stained liver sections. Scale bars = 100 μm.

FIGURE 6

TRF does not reduce systemic inflammation when consuming a HF diet.

Plasma IL-6 (A). Plasma TNF-α (B). Values are mean ± SEM, mice = 8-10 per group. *Chow *vs* HF, HF-early or HF-late ($P < 0.05$). (C) Representative FACS plot of eFl450™ stained $B220^{+}$

splenocytes gated $CD3^{neg}$ $B220^{+}$ cells after 72h of LPS stimulation at 37°C. ("min" (-) indicates negative control containing no LPS, "plus" (+) indicates addition of LPS) (D) Percentage proliferation of gated $B220^{+}$ cells (shown in (C)) after 72h of LPS stimulation. Data represent mean ± SEM for n=2 (Chow), n=3 (HF, HF-early, HF-late). LPS-induced proliferation is increased with *ad libitum* HF diet compared to all other groups *($P < 0.05$).

Feeding schedule

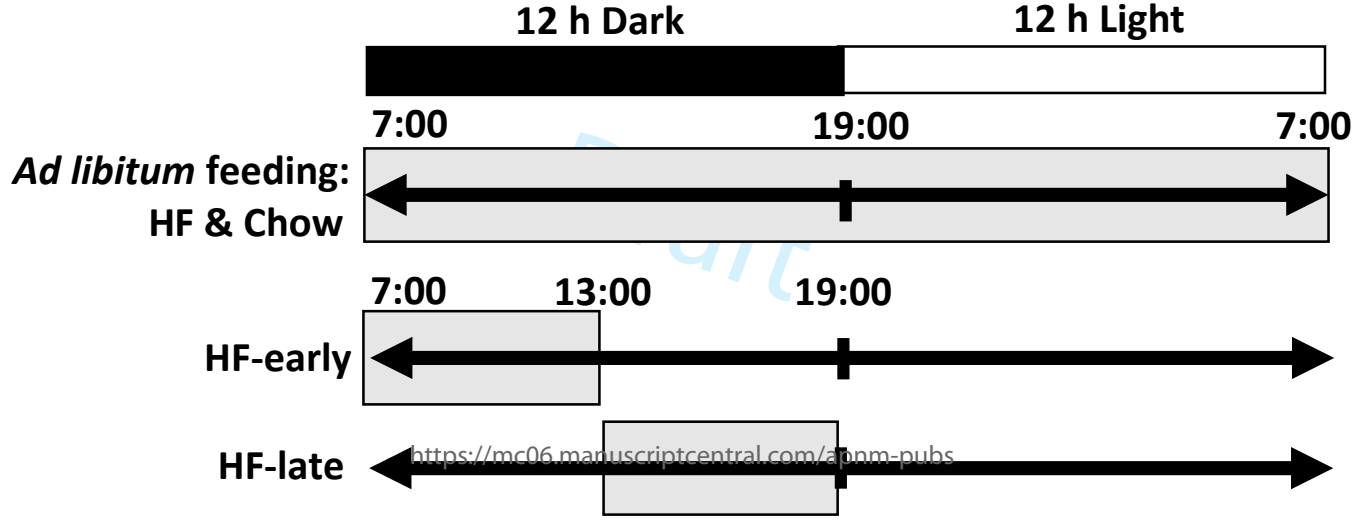


FIGURE 2

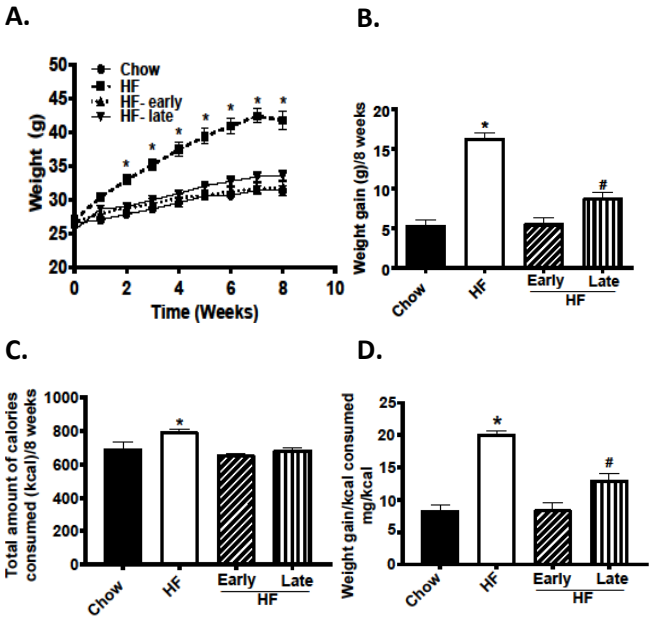


FIGURE 3

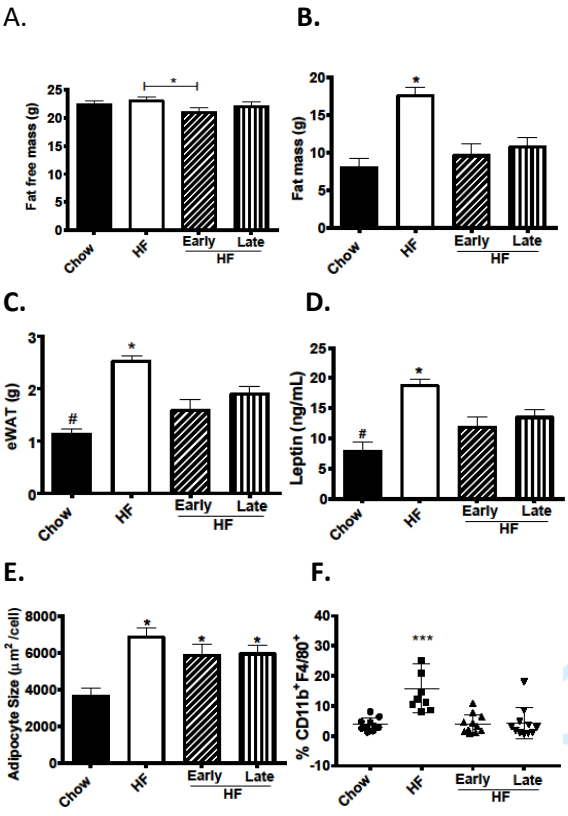
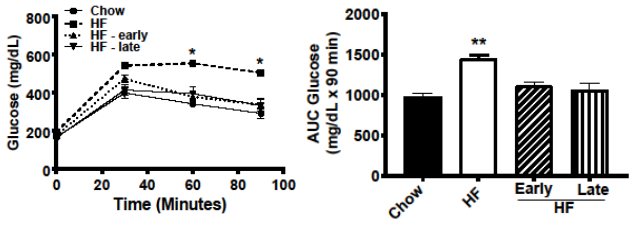


FIGURE 4

A.



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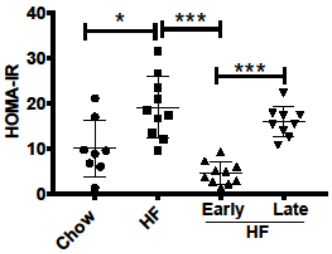
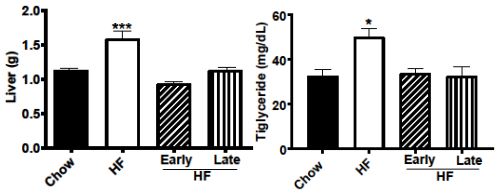


FIGURE 5

A. **B.**



C.

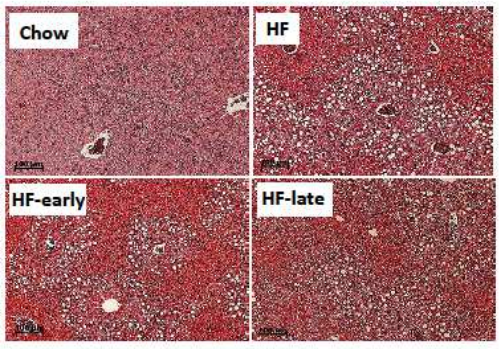


FIGURE 6