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RESEARCH PAPER

Time-restricted feeding does not prevent adverse effects of palatable cafeteria diet on adiposity, cognition and gut microbiota in rats

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Abstract

Time-restricted feeding (TRF) is a popular dietary strategy whereby daily food intake is limited to a <12h window. As little is known about the effects of TRF on cognitive and behavioral measures, the present study examined the effects of time-restricted (8h/day; zeitgeber time [ZT]12–20) or continuous access to a high-fat, high-sugar cafeteria-style diet (Caf; Caf and Caf-TRF groups; *n*=12 adult male Sprague-Dawley rats) or standard chow (Chow and Chow-TRF groups) on short-term memory, anxiety-like behavior, adiposity and gut microbiota composition over 13-weeks with daily food intake measures. TRF significantly reduced daily energy intake in Caf- but not chow-fed groups. In Caf-fed groups, TRF reduced the proportion of energy derived from sugar while increasing that derived from protein. Caf diet significantly increased weight gain, adiposity and fasting glucose within 4 weeks; TRF partially reduced these effects. Caf diet increased anxiety-like behavior in the Elevated Plus Maze in week 3 but not week 12, and impaired hippocampal-dependent place recognition memory in week 11; neither measure was affected by TRF. Global microbiota composition differed markedly between chow and Caf groups, with a small effect of TRF in rats fed chow. In both chow and Caf diet groups, TRF reduced microbiota alpha diversity measures of Shannon diversity and evenness relative to continuous access. Results indicate only limited benefits of TRF access to an obesogenic diet under these conditions, suggesting that more severe time restriction may be required to offset adverse metabolic and cognitive effects when using highly palatable diets.

Keywords: Time-restricted feeding; Cafeteria diet; Obesity; Rat; Anxiety; Memory; Microbiota; High fat diet.

1. Introduction

Time-restricted feeding (TRF) is a variant of intermittent fasting in which energy intake is confined to a set window of less than 12h each day [1]. A recent meta-analysis of human studies found that body weight and fasting glucose were reduced by TRF interventions lasting 4–8 weeks [2], and rodent models have shown that the adverse metabolic effects of high-fat/high-sugar diets can be ameliorated by TRF interventions [3–6], even when TRF is only implemented on weekdays [7] and without the need for reductions in energy intake [8]. The metabolic benefits of TRF are thought to be conferred by the alignment of food intake with circadian rhythms in metabolism [9] and are thus sensitive to the positioning of the TRF window across the light: dark cycle [10–13]. One study in rats found that the benefits of TRF on serum measures of oxidative stress and inflammation were comparable to those produced by alternate-day fasting [14].

While little research has specifically assessed behavioral impacts, it is important to identify how TRF alters mood, behavior and cognition, as these psychological factors may influence long term adherence to TRF [15] and thus any health outcomes. Evidence for any cognitive effects of TRF is mixed: Cross-sectional data from an Italian cohort suggested a reduced likelihood of cognitive impairment in participants self-reporting a daily eating window of less than 10 hours [16]. On the other hand, a 6-week TRF intervention (8hr/day) in healthy middle-aged adults did not alter cognitive function [17]. Results of the few studies using rodent models also vary, with one finding that a 12-hr/day TRF intervention prevented changes in anxiety- and depression-like behavior induced by sleep deprivation in rats but did not alter short-term recognition memory [18]. By contrast, another study [19] found that two weeks on a 12h/day TRF schedule following *ad-libitum* high-fat diet access restored spatial working memory, while beneficial effects of TRF on memory endpoints have been reported in diabetic db/db mice [20] and Alzheimer's disease models [21].

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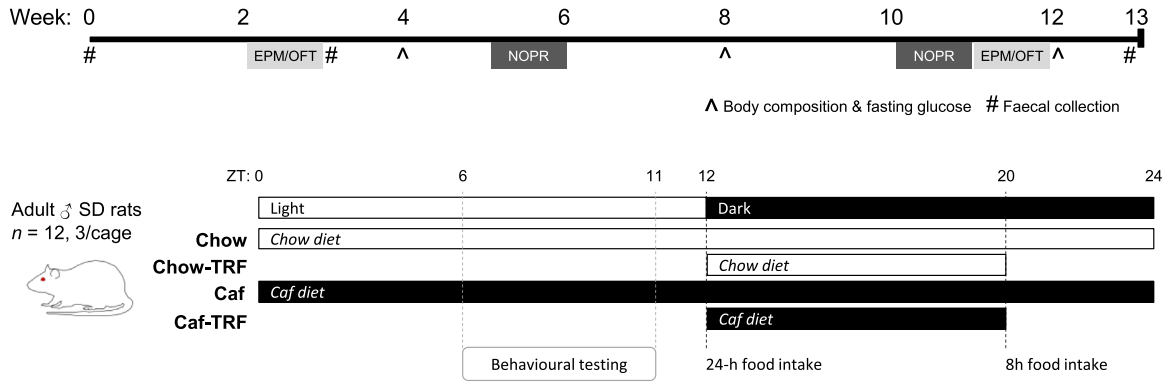

Figure 1. Experimental timeline and daily feeding schedule. Top: behavioral and metabolic measures were assessed across a 13-week diet intervention and body composition assessed in weeks 4, 8 and 12 by EchoMRI. Faeces were collected at baseline, week 3 and at endpoint. Anxiety-like behavior and cognition were tested after short- and long-term diet access. EPM, elevated plus maze; NOPR, novel object/place recognition; OFT, open field test. Bottom: daily 24-h feeding schedule. Food access for TRF groups began at ZT12 each day, when groups were transferred to a designated feeding cage. Food intake was measured in all groups at ZT20, when food access ended for TRF groups, and again at ZT12 the following day. Behavioral tests were conducted between ZT6-11.

Benefits of TRF on cognition were also observed in aged rats, where long-term maintenance on an extreme TRF schedule (~3hrs/day) of standard chow or a ketogenic diet reduced errors on a biconditional object discrimination task for food reward [22]. Finally, a 2h/day TRF schedule for five weeks increased locomotor activity in familiar and novel environments in young female rats [23].

Varied effects on behavioral and cognitive tests may relate to differences in the lengths of TRF interventions used. Moreover, few studies have tested whether the effects of TRF on cognition and behavior vary over time. To address this research gap, the present study assessed the effects of short- and long-term TRF on short-term memory (place and object recognition) and anxiety-like behavior (open field test and elevated plus maze) across a 13-week study in adult male rats fed either standard chow or an obesogenic high-fat, high-sugar cafeteria style (Caf) diet [24] that differs from the homogenized, purified high-fat diets commonly used. The experiment tests the hypothesis that TRF will prevent Caf diet-induced cognitive and metabolic impairments. Fasting glucose and body composition were measured in weeks 4, 8 and 12, and faecal samples were collected at week 0 (baseline), 3 and 13 to assess microbiota composition, which is sensitive to shifts in diet and TRF [25–27].

2. Methods

2.1. Animals and groups

All experimental procedures were approved by the Animal Care and Ethics Committee at UNSW Sydney (ethics approval 20/113A) and complied with the Australian code for the care and use of animals for scientific purposes 8th edition. Forty-eight adult male Sprague-Dawley rats were sourced from Animal Resources Centre (Perth, Australia) and housed three per cage in a temperature- and humidity-controlled vivarium maintained on a 12:12 light: dark cycle (lights on at 0100h, or Zeitgeber time 0 [ZT0]). During a week of acclimation rats were handled daily and weighed on alternate days, then allocated to four weight-matched diet groups (n=12) given *ad-libitum* access or time-restricted access (8hr/day; ZT12-20) to chow (14 kJ/g, 65% energy as carbohydrate, 22% protein and 13% fat; Chow and Chow-TRF) or a high-fat, high-sugar cafeteria-style (Caf; [24]) diet of palatable sweet and savory foods purchased from Coles® and Woolworths® supermarkets, in addition to chow and water (Caf and Caf-TRF). Each day, Caf and Caf-TRF groups were provided with a savory food (e.g., meat pie, dim sum, dog roll), and 3–4 other high-fat, high-sugar options including cakes (e.g., chocolate mud cake, jam sponge roll, lamington), biscuits (e.g., chocolate chip cookie, scotch fingers), purified high-fat chow (23.5% fat, Specialty Feeds, Glen Forrest, Western Australia) and 10% sucrose solution in addition to chow and water. A 10% sucrose solution approximates the concentration of most commercially available sugar-sweetened beverages and was only available during the 8-hr feeding window for the Caf-TRF group.

2.2. Feeding procedure

Figure 1 shows experimental procedures across the 13-week diet intervention and the 24-h feeding routine. Food intake was measured twice per day at the beginning (ZT12) and end (ZT20) of the feeding window for Chow-TRF and Caf-TRF groups. To confine food access to an 8-hour window for TRF groups and minimize disturbance of the rats during food intake measurement, at ZT12 rats in each cage were transferred to an identical, designated feeding cage where the forthcoming day's food was available. At ZT20 rats were returned to their original cage and food was collected, weighed to the nearest 0.1g, then either discarded (for TRF groups) or transferred with rats to the original cage (for continuous groups). The Caf diet was replenished daily for Caf and Caf-TRF groups, which received the same selection of foods each day.

2.3. Metabolic measures

Body weight was measured twice per week across the diet intervention. Whole-body fat and lean mass were quantified by EchoMRI (BRIL, Mark Wainwright Analytical Centre, UNSW Sydney) at baseline and in weeks 4, 8 and 12. On the day after EchoMRI measures in weeks 4, 8 and 12, food was removed at ZT20 for all groups and fasting blood glucose was measured from the tail tip with a glucometer (AccuChek Performa) the following morning (ZT7-8). Fasted rats were humanely euthanized over 4 days in week 13 (12 rats/day, n=3/group, ZT7-11). After intraperitoneal injection of ketamine/xylazine, body weight, girth and naso-anal length were measured; rats were then decapitated and liver, retroperitoneal fat and brown adipose tissue were excised and weighed. Commercial kits were used to analyses glucagon (Mercodia ELISA 10-1281-01, Uppsala, Sweden), folate (Folate Accubind ELISA 7525-300B, Lake Forest, California, USA) and insulin (Crystal Chem Ultra-Sensitive Rat Insulin ELISA kit 90060, Elk Grove Village, Illinois, USA) in fasted plasma at kill.

2.4. Cognitive and behavioral tests

Cognitive and behavioral tests were conducted in a dedicated test room between ZT6-ZT11. Anxiety-like behavior was tested in the elevated plus maze (EPM) and open field (OF) tests in weeks 3 and 12, with data scored automatically using ANY-maze software. EPM and OF tests were separated by 2–4 days and their order was counterbalanced within groups. The EPM consisted of two open and two closed arms (each 50cm × 10cm, approx. 200 lux and 15 lux, respectively) intersecting at a central platform, elevated 50cm above the ground. Rats were placed in the central square facing an open arm and allowed to explore for 5min. The key measures were entries into, and time spent in the closed and open arms, plus locomotor activity. The OF apparatus was a large square arena (120×120cm) brightly lit from above (240 lux), divided virtually into a 4×4 grid that was used to score time spent in the central four squares, outer 12 squares, and four corner squares.

Place and object recognition memory was assessed in a black acrylic arena (60×60cm) in weeks 6 and 11 of the diet intervention. After two 5-min habituation sessions in the empty arena rats underwent place and object recognition memory in a counterbalanced order, with a day separating the two tests. Both tests began with a 5-min familiarization phase in which rats explored two identical objects in the arena, followed by a 5-min retention phase where rats were returned to the home cage and objects and the arena were cleaned with 50% ethanol. In the final 3-min test phase rats were returned to the arena containing one original and one new object (*object* recognition) or both original objects but with one now in a novel location (*place* recognition). A video camera mounted overhead recorded behavior and trained scorers "blind" with respect to group scored object exploration during the test phase to calculate a *recognition index*: time exploring the novel or novel-located object (s) / total object exploration time (s), wherein values above 0.5 reflect preferential exploration of the novel object and thus intact memory.

2.5. Faecal microbiota composition

During the first week after arrival, faeces collected from each cage were mixed and redistributed across cages to promote standardization of the microbiome. Faecal samples were collected at baseline (one week after arrival, when all rats were on chow) and during week 3 of the diet intervention. These two samples were collected when measuring body weight and snap-frozen on dry ice in a sterile microtube. At endpoint (week 13) a third faecal sample was collected from the distal colon and snap-frozen in liquid nitrogen. Faecal DNA was extracted (DNeasy PowerSoil Pro kit, Qiagen, 47016) and, after assessing concentration and quality (DeNovix DS-11, DeNovix, USA), underwent Illumina sequencing at the Ramaciotti Centre for Genomics at UNSW Sydney (2×250bp MiSeq, V4 region, 515F-806R primer pair). Mothur (v.1.42.3) was used to process the raw sequence data with commands modified from the MiSeq SOP [28] to align with the SILVA database (v132), check chimeras with vsearch (v2.13.3), remove singletons and classify against the RDP training set (version18_032020). Sequence data were subsampled to n=6,430 clean reads per sample. No faeces were available from one rat in the Chow-TRF group at week 13, leaving n=11 for this group at this time.

2.6. Statistical analysis

All results are expressed as means ± standard error of the mean (SEM) and were considered significant when $P < .05$. Data were analyzed with IBM SPSS Statistics (v26, Armonk, New York, USA) using two-way ANOVAs (diet [chow or Caf] x access [continuous or TRF]), followed by post-hoc pairwise comparisons, where appropriate, applying the Tukey correction to control type-1 error rate. Mixed-ANOVAs were applied to assess changes over time. As liver scores were made on an ordinal scale from 0 (healthy) to 3 (unhealthy), nonparametric Kruskal-Wallis test followed by post-hoc Kolmogorov-Smirnov pairwise tests were used for this measure. Primer (v7; Albany, Auckland, New Zealand [29]) was used to calculate microbiota alpha diversity measures and relative abundances, which were subjected to a square root transformation in order to construct a Bray-Curtis similarity matrix at the OTU level. Permutational multivariate ANOVA (PERMANOVA) and non-metric multidimensional scaling plots were used to analyses group differences and temporal changes in microbiota beta diversity; alpha diversity measures were analyzed by mixed-ANOVA including cage as a covariate.

3. Results

3.1. Energy intake

Energy intake (Figure 2A and B) was significantly greater in Caf than Chow groups (Caf diet main effect: F [1, 12]=237.61, $P < .001$) and lower in TRF groups (access main effect: F [1, 12]=58.75, $P < .001$) with a significant diet x access interaction (F [1, 12]=21.89, $P < .001$). Follow-up pairwise comparisons indicated that TRF significantly reduced daily energy intake in Caf-fed groups (28% reduc-

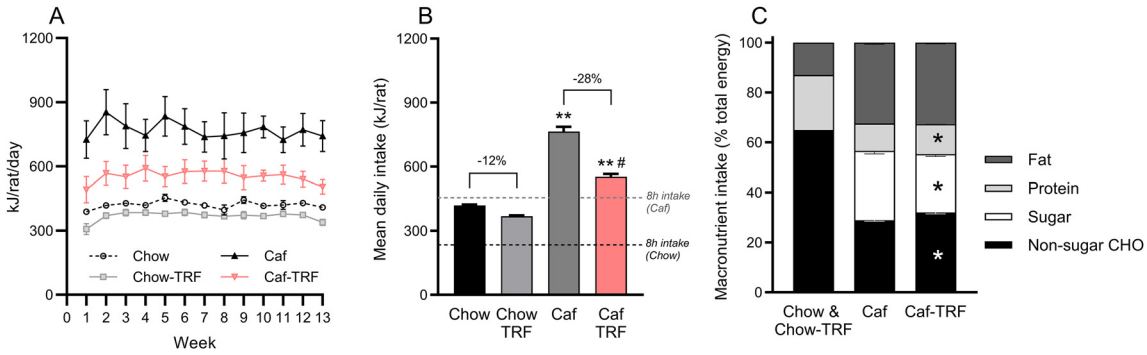

Figure 2. Energy and macronutrient intake in rats with continuous or time-restricted access to a high-fat, high-sugar "cafeteria" diet or standard chow. (A) Energy intake was stable across groups (B) average energy intake was significantly elevated by Caf diet (** $P < .01$ vs. Chow groups, Tukey post-hoc). TRF significantly reduced energy intake in rats fed Caf (# $P = .001$ vs. Caf). Dotted lines on y-axis represent 8-h intake by continuous groups (C) TRF altered the proportions of energy derived from sugar, nonsugar carbohydrates and protein (* $P < .05$ vs. Caf), with composition of chow shown for comparison. Data are means ± SEM; analyzed by mixed-ANOVA followed by Tukey post-hoc pairwise comparisons; $n = 4$ cages/group.

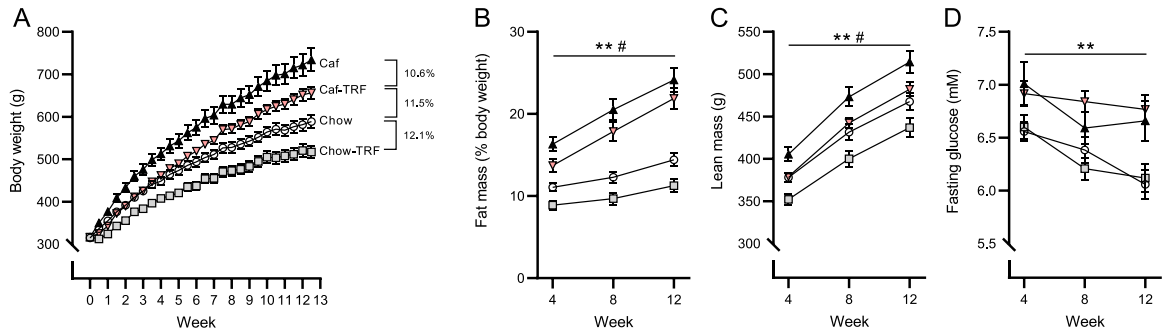

Figure 3. Body composition and fasting glucose in rats given continuous or time-restricted access to a high-fat, high-sugar "cafeteria" diet or standard chow. (A) Caf diet increased and TRF decreased body weight gain, respectively, with all groups significantly different at endpoint. Caf diet increased and TRF decreased percent fat mass (B) and net lean mass (C) whereas fasting blood glucose was elevated in Caf-fed groups with no effect of TRF (D) Data shown as means ± SEM; analyzed by mixed-ANOVA; $n = 12$. $^{**}P < .001$, main effect of Caf diet; # $P < .01$, main effect of TRF.

tion in Caf-TRF vs. Caf; $P < .001$) but the difference was not statistically significant in Chow-fed groups (12% reduction in Chow-TRF vs. Chow; $P = .204$; Tukey post-hoc). There were no significant changes over time across the 13-week diet intervention (Figure 2A; linear trend and interactions; largest F [1, 12]=3.96, $P = .07$). Figure 2C shows macronutrient intake for the two Caf-fed groups alongside the fixed macronutrient composition of chow. Relative to the continuous Caf group, the Caf-TRF group derived a significantly greater proportion of energy from protein (F [1, 6]=9.80, $P = .02$) and nonsugar carbohydrate (F [1, 6]=15.68, $P = .007$) and significantly less energy from sugar (F [1, 6]=7.00, $P = .038$) with no difference in the proportion of energy derived from fat (F <1).

3.2. Body weight and metabolic measures

As shown in Figure 3A, body weight gain was significantly greater in Caf- than chow-fed groups (time x diet linear interaction: F [1, 44]=75.06, $P < .001$) and in continuous relative to TRF groups (time x access linear interaction: F [1, 44]=10.58, $P < .001$) with no three-way interaction (F <1). Terminal body weight was elevated in Caf groups (diet main effect: F [1, 44]=67.70, $P < .001$) and reduced in TRF groups, on average (F [1, 44]=10.06, $P = .003$) with no interaction (F <1). Follow-up comparisons confirmed that TRF significantly reduced weight gain in groups fed chow (by 12.1%, $P = .041$) and Caf (by 10.6%, $P = .022$), but that endpoint body weight was significantly greater in group Caf-TRF relative to group Chow (by 11.5%, $P < .01$; Figure 3A).

Percent fat mass was significantly greater in Caf- than chow-fed groups (Figure 3B, diet main effect: F [1, 44]=72.72, $P < .001$), with the magnitude of the difference growing across the experiment (time x diet interaction, F [1, 44]=65.84, $P < .001$). TRF reduced percent fat mass in both diet groups (TRF main effect: F [1, 44]=7.87, $P = .007$) with no significant diet x TRF interactions (all $p > .05$). Net lean mass increased over time in all groups (Figure 3C, linear trend: F [1, 44]=1097.90, $P < .001$) and was significantly greater in Caf-fed groups (diet main effect: F [1, 44]=20.12, $P < .001$) and lower in TRF groups, on average (TRF main effect: F [1, 44]=11.95, $P = .001$) with no interaction (F <1). Fasting blood glucose (Figure 3D) was significantly elevated in Caf-fed groups (F[1, 43]=19.37, $P < .001$) with no effect of TRF (F <1) and an overall decrease across time (linear trend F [1, 43]=17.85, $P < .001$).

3.3. Elevated plus maze

Figure 4 shows the distribution of time (top panels) and open arm entries and locomotor activity (bottom panels) in EPM tests held in weeks 3 and 12. In week 3, Caf groups made significantly fewer entries into the open arms (F [1, 44]=4.23, $P = .046$, Figure 4 lower panel) though no significant differences were found for open arm time (F [1, 44]=2.80, $P = .10$), closed arm time (F [1, 44]=2.92, $P = .09$) or center square time (F [1, 44]=1.25, $P = .27$; Figure 4 upper panel). TRF increased locomotor activity in Caf groups and decreased locomotor activity in chow groups (diet x TRF interaction: F [1, 44]=7.03, $P = .011$). At week 12 there were no significant group differences in any EPM measures (largest F [1, 44]=2.98, $P = .09$). Relative to the week 3 test, at week 12 there were greater open arm entries (F [1, 44]=6.88, $P = .012$), open arm time (F [1, 44]=15.87, $P < .001$), center square time (F [1, 44]=23.27, $P < .001$) and locomotor activity (F [1, 44]=23.31, $P < .001$) and lower closed arm time (F [1, 44]=34.38, $P < .001$). These changes over time did not interact with diet or TRF (largest F [1, 44]=1.77, $P = .19$).

3.4. Open field test

As shown in Figure 5, groups did not differ significantly in center, perimeter or corner time, nor in rearing or distance travelled, in either week 3 (largest F [1, 44]=2.69, $P = .11$) or week 12 (largest F [1, 44]=2.78, $P = .10$). Relative to the week 3 test, at week 12 there were significant reductions in locomotor activity (F [1, 44]=20.10, $P < .001$), corner time (F [1, 44]=44.80, $P < .001$) and rearing (F [1, 44]=11.15, $P = .002$) and significantly greater perimeter time (F [1, 44]=36.67, $P < .001$), with no change in center time (F <1) and no interactions with group (all F <1).

3.5. Place and object recognition memory

Figure 6 shows place and object recognition memory in weeks 6 and 11. After 6 weeks of diet there were no significant effects of Caf diet or TRF on place or object recognition (largest F [1, 44]=1.74, $P = .19$). At week 11, place recognition was significantly lower in Caf-fed groups (Caf main effect: F [1, 44]=4.64, $P = .037$) with no TRF main effect (F <1) and no interaction (F [1, 44]=2.82, $P = .10$), and no group differences in object recognition (all F <1). There were no significant differences in performance between week 6 and week 11 tests (largest F [1, 44]=1.54, $P = .22$) and no differences in total object exploration time on any test (all F <1).

3.6. Endpoint measures

Table 1 shows anthropometric and metabolic measures after 13 weeks of continuous or time-restricted access to chow or Caf di-

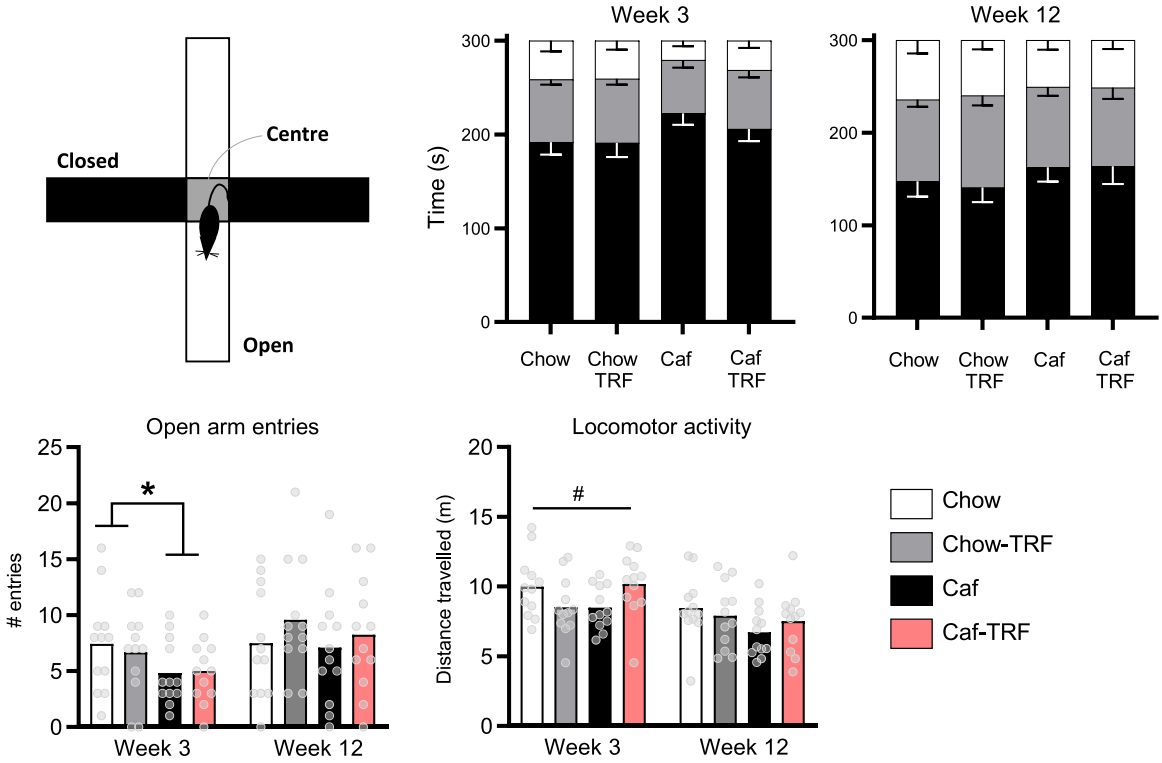

Figure 4. Elevated plus maze behavior in rats given continuous or time-restricted access to a high-fat, high-sugar "cafeteria" style diet or standard chow. Apparatus was divided virtually into open arm, closed arm and center square regions for analysis. Top panel: time spent in the open arms (white), closed arms (black) and the center square (grey) of the apparatus did not differ significantly between groups at either test. Bottom panel: Caf diet reduced open arm entries at week 3 ($^*P < .05$) and locomotor activity was altered by a Caf x TRF interaction (# $P < .05$). Data shown as means ± SEM; analyzed by mixed-ANOVA; $n = 12$.

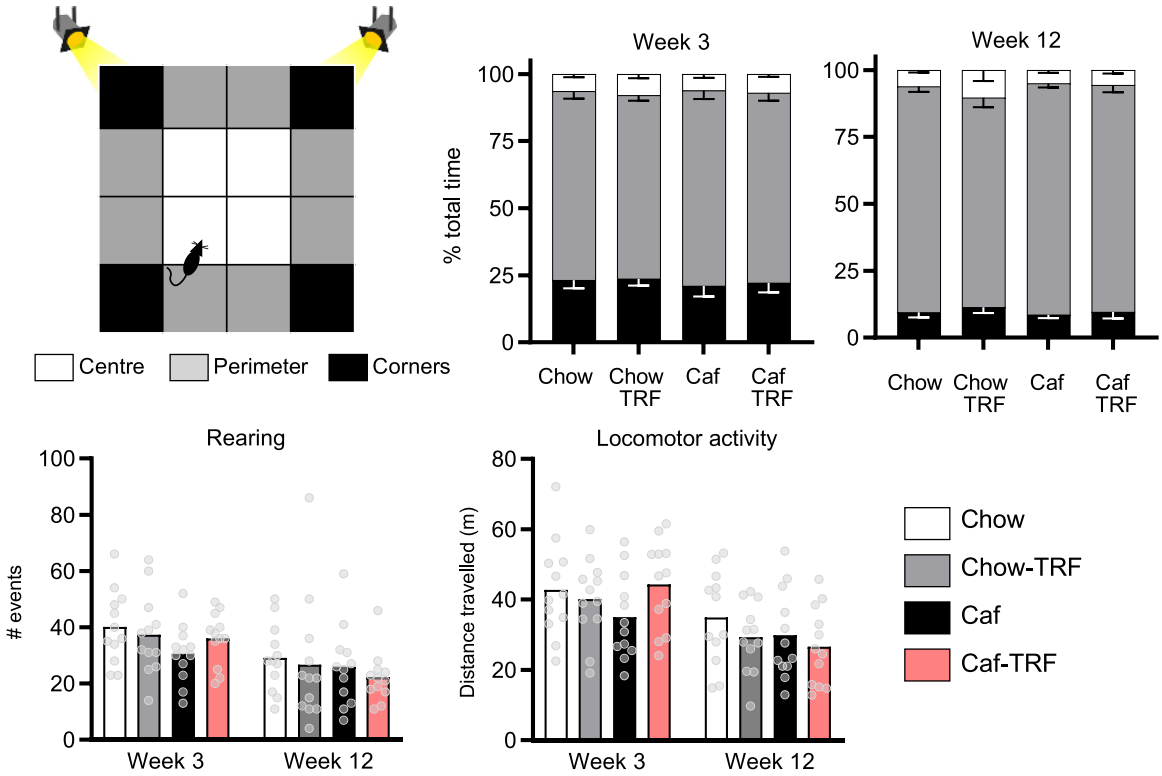

Figure 5. Open field test behavior in rats given continuous or time-restricted access to a high-fat, high-sugar "cafeteria" style diet or standard chow. Apparatus was virtually divided into center, perimeter and corner regions for analysis. Top: Groups did not differ in the proportion of time in the center (white), corners (black) or perimeter (grey) of the arena on either test. Bottom: rearing and locomotor activity did not differ between groups. Data shown as means ± SEM; analyzed by mixed-ANOVA; $n = 12$.

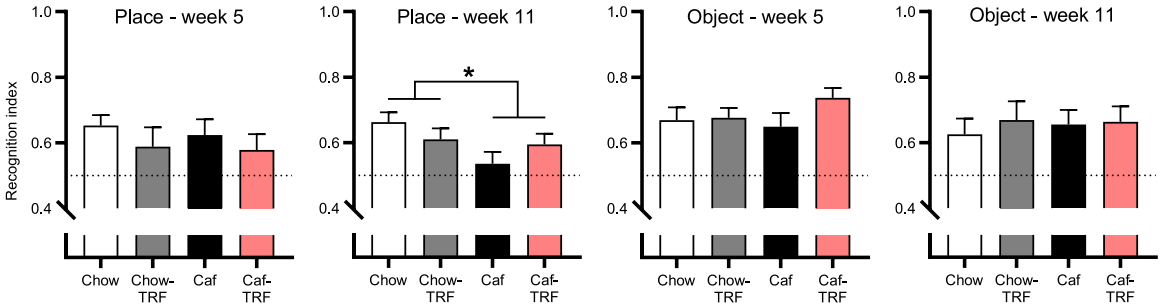

Figure 6. Place and object recognition in rats given continuous or time-restricted access to a high-fat, high-sugar "cafeteria" style diet or standard chow. Place recognition was impaired by Caf diet after 11 but not 6 weeks of diet (*$P < .05$, Caf diet main effect). There were no group differences in object recognition on either test. Data shown as means ± SEM; analyzed by mixed-ANOVA; $n = 12$.

Table 1
Metabolic and physiological effects of time-restricted or continuous access to chow and Caf diets.

Measure	Chow	Chow-TRF	Caf	Caf-TRF	2×2 ANOVA *p*-values (df 1, 44)		
					Caf diet main effect	TRF main effect	Caf x TRF interaction
Body weight (BW; g)	575.0 (14.5)	520.8 (14.7)	728.5 (26.5)	667.8 (14.4)	**<.001**	**0.003**	0.843
Naso-anal length (cm)	25.8 (0.20)	25.4 (0.25)	27.0 (0.21)	26.6 (0.14)	**<.001**	**0.047**	0.999
Girth (cm)	20.0 (0.3)	19.0 (0.3)	22.9 (0.37)	21.9 (0.35)	**<.001**	**0.002**	0.902
Liver score (0-3)	0.33 (0.2)^{A}	0.17 (0.1)^{A}	2.67 (0.14)^{B}	1.91 (0.19)^{B}	**<.001** (omnibus Kruskal Wallis test)		
Liver weight (g)	15.78 (0.77)	14.68 (0.78)	22.15 (1.72)	18.78 (0.99)	**<.001**	0.056	0.318
Liver weight (% BW)	2.72 (0.08)	2.80 (0.09)	3.00 (0.12)	2.80 (0.11)	0.187	0.398	0.175
RP WAT (g)	10.3 (1.0)	8.1 (0.8)	26.6 (2.8)	21.8 (1.8)	**<.001**	0.055	0.464
RP WAT (% BW)	1.76 (0.13)	1.54 (0.13)	3.57 (0.26)	3.23 (0.24)	**<.001**	0.166	0.761
BAT (g)	0.48 (0.04)	0.35 (0.04)	0.81 (0.04)	0.74 (0.07)	**<.001**	0.072	0.598
Plasma folate (ng/mL)	64.06 (2.82)	63.13 (5.23)	39.64 (3.60)	39.33 (3.58)	**<.001**	0.878	0.938
Plasma glucagon (pmol/L)	54.74 (5.39)	48.39 (6.18)	22.90 (2.33)	29.12 (3.01)	**<.001**	0.524	0.168
Plasma insulin (ng/mL)	1.02 (0.20)	1.29 (0.37)	1.79 (0.24)	2.74 (0.62)	**<.001**	0.142	0.406

Data shown as means ± SEM.
Abbreviations: BAT, brown adipose tissue; RP, retroperitoneal; WAT, white adipose tissue.
n=12, except plasma folate and glucagon n = 10–12.
N.B. For liver scores, groups not sharing a letter ("A" and "B") differ significantly ($p < .05$) from post-hoc pairwise Kolmogorov-Smirnov tests.

ets. Significant Caf diet main effects were found for all measures except weight-adjusted liver mass. TRF significantly reduced body weight, naso-anal length and girth, on average, with a marginal effect for retroperitoneal fat mass. No Caf x TRF interaction effects were significant.

3.7. Faecal microbiota composition

3.7.1. Alpha diversity

Changes in microbiota species richness (Figure 7A) differed according to a time x diet interaction (F [2, 84]=7.90, $P < .001$) with no other significant main or interaction effects. Subsequent analyses found no group differences in richness at baseline (week 0; all F <1), significantly lower richness in Caf groups at week 3 (Caf diet main effect: F [1, 43]=4.78, $P = .034$), but significantly greater richness in Caf groups at week 13 (Caf diet main effect: F [1, 42]=5.63, $P = .022$) driven by a steep reduction in chow groups. There were significant time x TRF interactions for Shannon diversity (Figure 7B; F [2, 84]=7.06, $P = .001$) and Pielou's evenness (Figure 7C; F [2, 84]=8.98, $P < .001$). Both measures were significantly lower in TRF groups at week 13 (TRF main effects: Shannon diversity F [1, 42]=11.65, $P = .001$; evenness F [1, 42]=17.39, $P < .001$) with no group differences at weeks 0 or 3 (all $P > .13$). Home cage was included as a covariate with no significant effects of this factor observed.

3.7.2. Beta diversity

Nonmetric multidimensional scaling plots of global microbiota composition at weeks 0, 3 and 13 are shown in Figure 8A and C. Changes in microbiota composition over time were assessed by PERMANOVA (999 permutations) with factors of time (weeks 0, 3 and 13), diet (chow or Caf) and access (continuous or TRF). Analyses indicated significant main effects of time (pseudo-F=9.13, $P < .001$), diet (pseudo-F=20.72, $P = .001$) and access (pseudo-F=2.27, $P = .003$) as well as a time x diet interaction (pseudo-F=7.46, $P = .001$) but no other interactions. Separate PERMANOVA analyses were then run at each timepoint to identify the source of the interaction. While there were no significant group differences at baseline (week 0), chow and Caf-fed groups differed significantly at both weeks 3 and 13 (diet main effects; pseudo-F=16.76, $P = .001$ and pseudo-F=20.57, $P = .001$, respectively). There were also significant differences between continuous and TRF groups at week 3 (access main effect: pseudo-F=1.72, $P = .032$) and week 13 (pseudo-F=1.92, $P = .01$), with no diet x access interactions (all $P > .05$). Permutational Multivariate Analysis of Dispersion (PERMDISP) analyses found no significant differences in group dispersion at any time point (all $P > .096$).

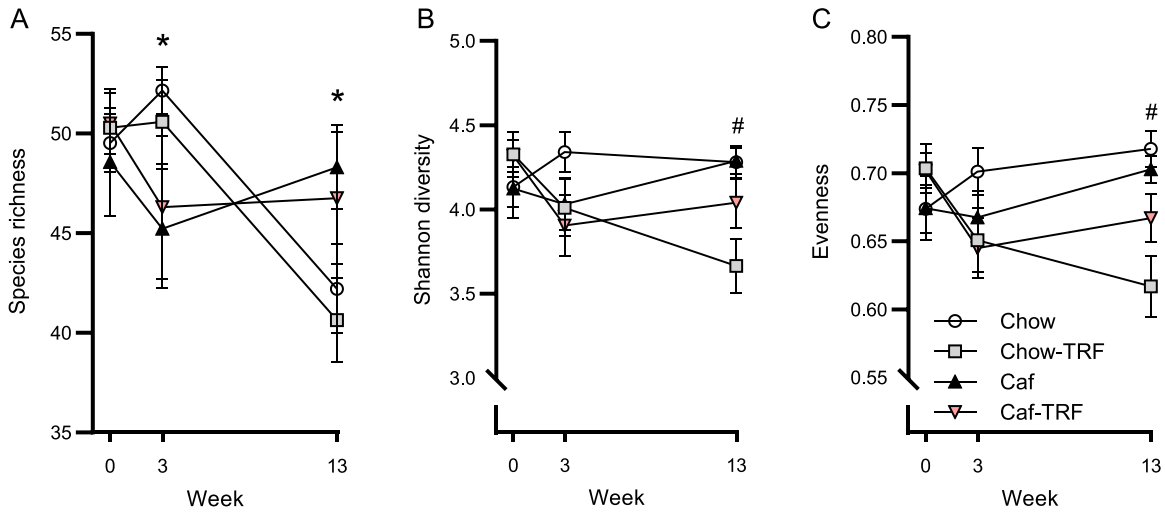

Figure 7. Faecal microbiota alpha diversity measures in rats given continuous or time-restricted access to a high-fat, high-sugar "cafeteria" diet or standard chow. (A) species richness was lower in Caf groups at week 3, but lower in chow groups at week 13 (B) Shannon diversity was lower in TRF groups at week 13 (C) Pielou's evenness was lower in TRF groups at week 13. * Caf diet main effect ($P < .05$). # TRF main effect ($P < .05$). Data shown as means ± SEM; analyzed by mixed-ANOVA; n=12 (Chow, Caf, Caf-TRF) and n=11 (Chow-TRF).

4. Discussion

Time-restricted feeding confers beneficial metabolic effects, but its impact on behavior and cognition is unclear. The present study is the first, to our knowledge, to examine the short- and long-term effects of time-restricted access to a healthy or obesogenic diet on anxiety-like behavior and short-term memory, markers of metabolic health and faecal microbiota composition. TRF had no detectable impact on behavior or cognition despite reducing body weight gain and adiposity, and altering microbiota composition in both diet groups. An important finding of this study is that while the obesogenic Caf diet increased anxiety-like behavior over the short-term and impaired spatial memory after long-term exposure, neither of these effects were offset by TRF; implications of these results are elaborated below.

Daily food intake measures indicated that 8h/day TRF decreased energy intake by 12% in chow-fed and 28% in Caf-fed cages, relative to groups fed *ad-libitum*, which was stable over time. That TRF led to a larger proportional reduction in intake for rats fed Caf is consistent with our past evidence that unrestricted Caf diet access promotes "snacking" during the light cycle [30], which was precluded by TRF access in the present study. Nonetheless, energy intake by Caf-TRF rats remained well above that of both chow-fed groups. An advantage of using a varied cafeteria-style diet with multiple palatable choices is that rats could self-select foods, providing an opportunity to assess macronutrient composition changes, recently argued to be an important outcome in TRF studies [31]. The present results suggest that as well as reducing total energy intake, time-restricted access to Caf diet altered macronutrient composition by decreasing the proportion of energy derived from sugar and increasing the proportion of energy derived from protein, suggesting that the Caf-TRF group prioritized intake of nonsweet, protein-rich foods, arguably a modest improvement in nutritional quality.

The TRF intervention had significant effects on body weight and adiposity measures that were remarkably consistent in chow- and Caf-fed groups. Thus, relative to their counterparts fed *ad-libitum*, TRF groups fed chow and Caf had significantly lower terminal body weight (~10% lower), girth (~5% lower), retroperitoneal fat (~20% lower) and whole-body adiposity (~10% lower). However, the beneficial effects of TRF were overshadowed by the obesogenic effects of the cafeteria diet, with the Caf-TRF group exhibiting an intermediate metabolic phenotype that was improved somewhat relative to the continuous Caf group but still poorer than chow controls. In fact, the Caf-TRF group was indistinguishable from the continuous Caf group on measures of fasting glucose and plasma insulin (increased vs. chow) and plasma glucagon and folate (decreased relative to Chow). As the marginal improvements in metabolic parameters produced by TRF in Caf-fed rats were not commensurate with the reduction in energy intake (~30% reduction vs. continuous Caf), examining differences in general activity, fat oxidation and other measures of energy expenditure may be fruitful in future work. The present result also contrasts previous studies showing that 8–12h TRF schedules can normalize weight gain to control levels when purified high-fat/high-sugar diets are used [4,8]. The present results suggest that shorter TRF windows (e.g., 4–6h) may be necessary to prevent metabolic impairment when using a varied, highly palatable obesogenic diet, which are more effective in promoting hyperphagia and obesity [32], and more indicative of the diet consumed by humans. However, using a similar design to this experiment, another study [3] found that 8h TRF access to a Caf-style diet fully prevented gains in body weight and adiposity, and increased expression of uncoupling protein 1 and PGC1a in inguinal WAT. These differences could be due to differences between the cafeteria diets used; for example, the diet used by Aouichat and colleagues contained several savory crisps and meat products that were not used here. Our study also differed in its use of group-housed rats, whereas rats were individually housed in Aouichat et al. [3].

In short-term memory tests, Caf-fed groups exhibited poorer place recognition after 11 weeks of diet, with no differences in object recognition, suggesting a specific impairment in hippocampal-dependent memory, consistent with previous work [33,34]. This deficit was not rescued by TRF, with comparable performance in continuous and time-restricted Caf groups. Cognitive and behavioral tests were administered during the light cycle as we reasoned that testing during the dark cycle, when food was available for TRF groups, might obscure results through entrainment effects; that is, a greater increase in general activity in TRF groups due to the feeding schedule. Nonetheless, results of a recent study [19] suggest that testing during the light cycle may have obscured cognitive effects of TRF. In that study, hippocampal-dependent mem-

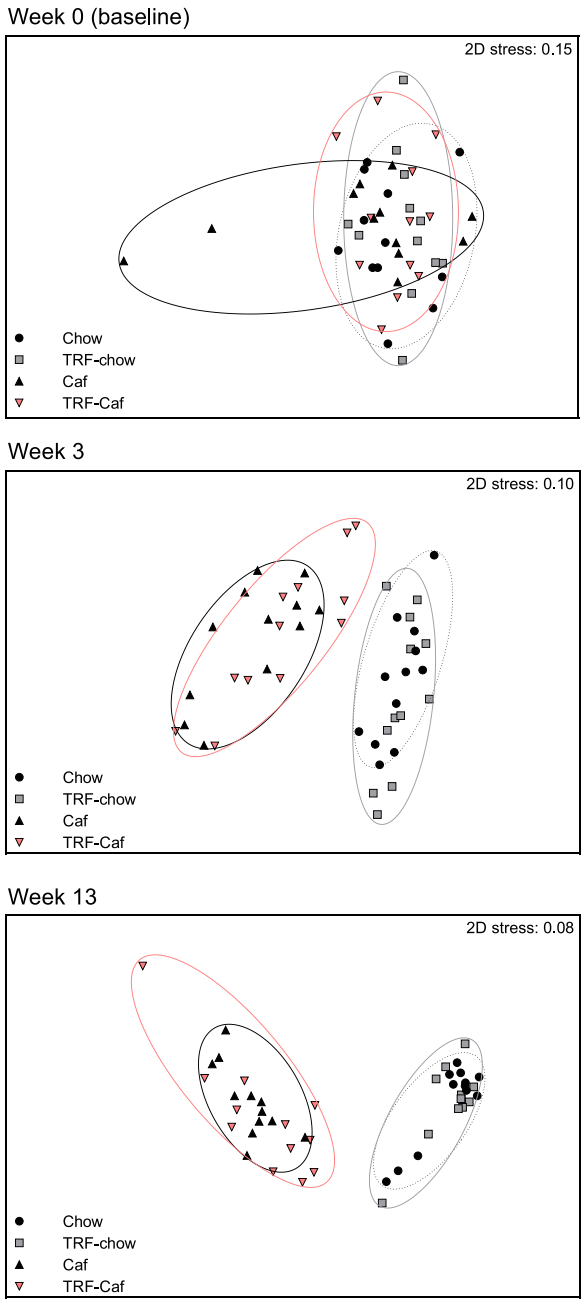

Figure 8. Nonmetric multidimensional scaling plots showing microbiota composition at baseline (A) week 3 (B) and week 13 (C) in rats given continuous or time-restricted access to chow or "cafeteria" diets. Permutational multivariate analysis of variance (PERMANOVA) showed significant main effects of diet (chow vs. Caf) and access (continuous vs. TRF) at weeks 3 and 13, with no interactions. NMDS plots derived from a Bray–Curtis similarity index at the OTU level, n=11–12. Data points represent individual rats; proximity reflects microbiota similarity.

ory (assessed by spontaneous T-maze alternation) was tested during the light and dark cycles in mice fed continuous or TRF access to control or high-fat diets. The key result was that testing during the dark cycle significantly improved spontaneous alternation in the TRF-HFD group (and chow groups), but not in the continuous HFD group, which exhibited poor performance in both cycles. This result suggests that testing during the dark cycle might have revealed a difference between Caf-TRF and continuous Caf groups; however, other studies have found no effects of TRF on novel object recognition when testing in the dark cycle [18]. Further work is required to assess whether diurnal fluctuations in hippocampal-dependent memory are test-specific (i.e., to T-maze alternation but not place/object recognition). Another possibility is that cognitive impairment would be prevented only by a TRF schedule that fully normalized body weight and adiposity changes, which was not the case in the present study.

TRF also had no detectable effects on anxiety-like behavior as measured in the EPM and OF tests in weeks 3 and 12. Instead, Caf diet led to an overall increase in anxiety-like behavior on the EPM at the week 3 test through decreased open arm entries and a nonsignificant trend toward increased closed arm time, consistent with a recent meta-analysis showing significant anxiogenic effects of obesogenic diets in rat and mouse studies [35]. However, in the present study no effects of Caf diet were observed on the OFT, where a strong preference for the perimeter of the arena persisted in all groups. In studies using varied "cafeteria"-style diets of the kind used here, results are variable, with anxiety-like behavior shown to be decreased [36–38], increased [39–41], altered in sex-specific ways [42,43] or unaffected by cafeteria diet [44].

Locomotor activity in both EPM and OFT decreased significantly from week 3 to week 12, and was not moderated by diet type, suggesting that all groups habituated to the tests. Importantly, however, both apparatuses retained their aversive properties at week 12, suggesting they remained valid assays of anxiety-like behaviour (see [45]). Here we observed no changes in locomotor activity in TRF groups, which consumed significantly less energy than their counterparts fed *ad-libitum*, in contrast to the meta-analysis by Clark and colleagues [35], which found some evidence that caloric restriction may increase activity, albeit based on a small number of studies.

TRF and related caloric restriction diets can produce beneficial or maladaptive changes in the gut microbiome, depending on the extent of restriction and other factors [46]. Here, TRF reduced gut microbiota Shannon diversity and evenness after 13 weeks, regardless of diet type, consistent with some past studies [47] whereas others have seen modest increases [48]. By contrast, species richness was unaffected by TRF but was ultimately lower in chow-fed groups. A recent systematic review [49] found mixed effects of TRF on alpha diversity in rodent models; differences in sample collection time appear a likely source of this variability in light of evidence for diurnal fluctuations in species abundance (e.g. [50]). In terms of global microbiota composition (beta diversity), here the effects of TRF, while still statistically significant, were modest in comparison to the substantial shifts induced by cafeteria diet exposure.

When designing animal models of TRF and other interventions centered on restricted feeding it is relevant to consider to what extent these align with organisms' natural feeding patterns. The 8–h TRF window used in the present study began just prior to the onset of the dark (active) phase, thus spanning the period when rodents typically consume the majority of their daily food intake. However, studies of diurnal feeding patterns show that both domesticated and wild rats eat appreciable amounts of food across the light phase [51] and our past work has shown that the palatable high-fat, high-sugar cafeteria diet used here promotes increased intake during the light cycle [52]. Consistent with this evidence, the TRF intervention here significantly reduced total energy intake and altered macronutrient composition in Caf diet groups. The 12% difference in energy intake between Chow and Chow-TRF groups, though not statistically significant, still led to significantly lower body weight, fat and lean mass in the Chow-TRF group. Effects of TRF on growth in chow groups appeared confined to the first 4 weeks of the intervention, since body composition data (see Figure 3C) suggested comparable lean mass gain in all groups from weeks 4 to 12. Similarly, linear growth measured at endpoint indi-

cated a modest effect of TRF on naso-anal length in chow groups, with a mean difference of 0.4cm (25.8 vs. 25.4 in Chow vs. Chow-TRF groups).

Limitations of our study include the use of only male rats, with sex-specific effects of TRF on metabolic outcomes observed in recent reports [5], and analysis of microbiota composition via faecal samples collected within a narrow temporal window, potentially missing more dynamic diurnal changes observed elsewhere in the gastrointestinal tract [50]. In light of evidence that TRF alters the lipidome in high-fat diet-fed mice [53], more detailed analysis of lipid metabolism appears warranted in this model, where TRF improved but did not normalize adiposity in Caf rats. In summary, the present study finds few benefits to cognitive and metabolic parameters following time-restricted access to an obesogenic diet in rats. Results suggest that TRF confers only limited benefits when highly palatable diets are consumed, suggesting that nutritional composition is an important factor to consider when modelling the effects of TRF and other variants of intermittent fasting in humans.

Declarations of interest

None.

CRediT authorship contribution statement

Margaret J. Morris: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Kyoko Hasebe:** Writing – review & editing, Investigation, Formal analysis. **Arya L. Shinde:** Writing – review & editing, Investigation. **Michael K. H Leong:** Writing – review & editing, Investigation. **Md. Mustahsan Billah:** Writing – review & editing, Investigation. **Sonia Hesam-Shariati:** Writing – review & editing, Investigation. **Michael D. Kendig:** Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

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