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Palatable western-style cafeteria diet as a reliable method for modelling diet-induced obesity in rodents

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Dear Dr Myers,

We are pleased to submit this revision of our manuscript, *Palatable western-style cafeteria diet as a reliable method for modelling diet-induced obesity in rodents*, and hope it is now suitable for publication in JoVE. We thank the reviewers for their comprehensive comments, which we believe have greatly improved our manuscript.

Sincerely,

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TITLE:

Palatable Western-Style Cafeteria Diet as a Reliable Method for Modeling Diet-Induced Obesity in Rodents

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KEYWORDS:

diet-induced obesity, cafeteria diet, energy intake, palatability, western diet, variety, hyperphagia, rodent

SUMMARY:

This protocol describes the use of a highly palatable, western-style cafeteria diet to model overeating and obesity in rodents. Here, we provide a detailed outline of food selection, preparation and measurement, and explain methodological factors that assist in generating a robust and reproducible phenotype.

ABSTRACT:

Obesity is rapidly increasing in incidence in developed and developing countries and is known to induce or exacerbate many diseases. The health burden of obesity and its comorbid conditions highlight the need for better understanding of its pathogenesis, yet ethical constraints limit studies in humans. To this end externally valid models of obesity in laboratory animals are essential for the understanding of being overweight and obesity. While many species have been used to model the range of changes that accompany obesity in humans, rodents are most commonly used. Our laboratory has developed a western-style cafeteria diet that consistently leads to considerable weight gain and markers of metabolic disease in rodents. The diet exposes rodents to a variety of highly palatable foods to induce hyperphagia, modeling the modern western food environment. This diet rapidly induces weight gain and body fat accumulation in rats allowing for the study of effects of overeating and obesity. While the cafeteria diet may not provide the same control over macronutrient and micronutrient profile as purified high-fat or high-fat, high-sugar diets, the cafeteria diet typically induces a more severe metabolic phenotype than that observed with purified diets and is more in line with metabolic disturbances observed in the overweight and obese human population.

INTRODUCTION:

Obesity and its related comorbidities make an enormous contribution to global health burden[1] and account for 7% of disease burden in Australia[2]. A leading risk factor for obesity is consumption of unhealthy diets that are high in saturated fat and refined carbohydrates, and low in fiber and micronutrients[3]. Identifying targets for therapeutic intervention for obesity requires models that can systematically assess effects on multiple biochemical and physiological systems. Our understanding of the etiology of obesity has been advanced substantially by work using rodent models, where behavioral, metabolic and molecular effects can be studied across time under controlled conditions where environmental factors can be easily manipulated.

The **cafeteria diet** (CAF) model of diet-induced obesity consists of supplementing rodents' standard chow diet with a variety of palatable foods that are high in either saturated fat, refined carbohydrates, or both. Examples of these foods include cakes, sweet biscuits, and high-fat savory snacks (such as processed meats, cheese and chips). It reliably promotes hyperphagia and rapid weight gain in rodents. The key features of the model are the provision of a **variety** of highly **palatable** foods, designed to simulate the modern food environment. Access to variety increases food intake in rats over the short-term[4] and in humans[5] even when the foods are matched for palatability and vary only in flavor and olfactory cues[4,6]. However, one study showed that providing energy- and macronutrient-matched purified diets that varied in flavor and texture had no effect on long-term body weight gain in rats[7], suggesting that nutrient composition and distinct post-oral effects of different foods may also contribute to overeating. Exposure to multiple tastes and textures overcomes sensory-specific satiety, which describes the decrease in desire to eat a recently eaten food relative to an alternative[5]. Across many cohorts in our laboratory, we have similarly observed that the use of highly palatable foods further amplifies overeating.

This CAF diet has been used for over 40 years, since Sclafani[8] reported that female rats exposed to an assortment of 'supermarket foods' (marshmallows, chocolate, peanut butter, cookies, salami and cheese among them) exhibited accelerated weight gain relative to controls. This and other early studies noted that CAF-style diets appeared to accelerate weight gain more effectively than pure high-fat or high-carbohydrate diets [8,9]. Work in the 1980s characterized the macronutrient profiles[10] and meal patterns[11] of rats fed CAF diets, and showed profound changes to fat mass and insulin levels[9,10] and thermogenesis[12]. Our group has used the CAF diet to model obesity for over two decades[13,14] and during this time we have used several variants of the diet. Rats are presented with at least two sweet and two savory food items each day, in addition to regular chow and water. In recent years we have begun to supplement solid CAF foods with 10% sucrose solution. The ability to tailor the CAF diet to different experimental designs is a strength of the model.

CAF diets promote immediate hyperphagia (i.e., within the first 24 h) and steady gains in body weight and fat mass. However, a consequence of maximizing variety is that macronutrient and micronutrient intake is not controlled, a point some view as an insurmountable flaw[15]. Studies of diet-induced obesity more commonly use purified high-fat (HF) or combined high-fat, high-sugar

(HFHS) diets, which offer precise control over nutritional content and are less labor-intensive than the CAF model, which requires daily monitoring and careful planning and execution of the schedule. The translational relevance of commercially available purified HF diets is a topic of ongoing debate, as their fatty acid profile and proportions of fat and sucrose may not align with human dietary intake[16]. While CAF diet does not offer the same degree of control over nutrient composition as purified diets, it aims to model the palatability and variety that characterizes food options in most modern societies.

PROTOCOL:

The protocol described here has been optimized for use in rats. While we have used the CAF diet successfully in mice[17,18], soft food grinding may introduce further error reducing the reliability of food intake measures[19]. This protocol is approved by the Animal Care and Ethics Committee at the University of New South Wales and complies with the Australian guidelines for the use and care of animals for scientific purposes (8th Edition) provided by the Australian National Health and Medical Research Council.

NOTE: Very few adverse effects have been observed in our short-term studies (i.e. <10 weeks ad libitum CAF access); there is no evidence of changes to general wellbeing, activity, sociability or anxiety-like behavior in rats on CAF diet[20]. After longer intervals (>16 weeks) very occasional cardiovascular incidents have been observed in CAF-fed rats.

1. Animal acclimatization and housing

1.1. Acclimate rats to the facility for 5–7 days after arrival with free access to control diet and water. Handle rats daily beginning 24 h after arrival; CAF diet involves daily interaction with the cage so regular handling is important.

1.2. Ensure environmental enrichment is provided to all cages. A standard cage contains a red polymethyl methacrylate box, nesting material and a wooden chew stick, which is important because the soft foods provided within the CAF diet may result in rats developing malocclusion without access to harder items to chew.

1.3. Prior to commencing CAF diet, assign cages to CAF or chow groups and ensure these are matched for starting body weight by comparing the mean and range of body weights between groups and re-allocating as necessary. As lighting exposure can affect circadian rhythms, food intake and activity, ensure CAF and chow cages are distributed evenly in the colony room.

NOTE: Rodents form social hierarchies when group-housed (especially males). The effects of social stress are partially controlled for by ensuring rats are housed with others of a similar body weight (to reduce bullying within a cage). Additionally, cafeteria diet should be evenly distributed around the cage so that all rats have access to the diet.

2. Diet selection and setup

2.1. Scheduling

2.1.1. Ensure regular chow and water are always available and are supplemented with a minimum of two sweet and two savory items each day (an example of a weekly schedule is shown in **Table 1**; food options for 3 days shown in **Figure 1**). Optional extras are high-fat, high-sugar chow and 10% sucrose solution.

2.1.2. Choose foods within each category that are similar in macronutrient proportions: all sweet items are relatively higher in carbohydrate than fat and all savory items are higher in fat than carbohydrate. Aim to provide similar proportions of fat, carbohydrate, sugar and protein in each daily set of CAF foods.

2.1.3. Regularly monitor consumption throughout the experiment so that the menu can be tailored, if necessary, to sustain hyperphagia. **Table 2** provides energy density and macronutrient information for several example foods, and suggested starting amounts for 200 g male Sprague Dawley rats.

2.1.4. Avoid feeding any single food on consecutive days or too often in a week, as this may decrease intake of that food. If this occurs, omit the food from the schedule for a few days before re-incorporating.

NOTE: Rats may display neophobia and most new foods require repeated exposures to determine whether they will be consumed. Rats do not typically prefer foods containing yeast (breads, pizza, etc.) or those with citrus or coffee flavors.

2.2. Wet foods

2.2.1. Consider presenting foods higher in moisture content in a container such as a tuna tin, to avoid soiling the bedding. Empty containers should also be provided to control cages if this is done.

2.3. Diet sourcing

2.3.1. Ensure that CAF foods used are staples or obtain enough for the whole experiment so that food items remain consistent and do not need to be substituted.

2.4. Diet storage

2.4.1. Store foods as indicated on the packaging or at -20 °C and thaw the night before use. Store dry goods (biscuits) in an airtight container.

2.5. Sucrose solution (optional)

2.5.1. Prepare sucrose solution, typically 10% (w/v) in bulk (2–5L) and store at 4 °C when not in

use.

2.5.2. Replace sucrose bottles weekly (at a minimum) to prevent bacterial or fungal growth. Inspect bottles daily and replace if signs of growth are observed.

NOTE: Sucrose is provided in addition to water, which is always available.

3. Cafeteria diet preparation

3.1. Defrost

3.1.1. Take out appropriate amounts of CAF foods to thaw ~24 h prior to use. Food can be defrosted using a microwave but should not be presented while hot.

3.2. Daily replenishing of CAF diet

3.2.1. Changing CAF diet foods daily ensures that variety is maximized, and cage soiling is minimized. As rats eat most of their daily intake in the initial portion of the dark cycle, schedule CAF food replenishment close to the onset of the dark schedule so that food is fresh at this time. Replenish CAF diet on a day when food intake is **not** being measured.

3.2.2. Feed the chow groups in a manner that ensures that this group has a similar daily experience, as follows.

3.2.2.1. Turn the water bottles around (spouts up), place the cage on the workspace and remove the lid.

3.2.2.2. Lightly disturb the bedding for approximately 20 s, to simulate the process of collecting food items from the bedding of CAF cages.

3.2.2.3. Place a small handful of chow pellets from the food hopper into the bedding, to equate exposure to food on the cage bedding.

3.2.2.4. Return the cage lid and return to the rack, replacing water bottles only when the cage is settled in order to minimize spillage.

3.2.3. Feeding the cafeteria groups

3.2.3.1. Prepare CAF diet items into a labeled container for each cage.

3.2.3.2. Turn the water bottles around (spouts up), place the cage on the workspace and remove the lid.

3.2.3.3. Remove as much of the old cafeteria diet from the bedding as possible.

3.2.3.4. Place fresh cafeteria diet into the cage.

3.2.3.5. Close the cage and return to the rack.

3.2.3.6. Replace the water and sucrose bottles.

NOTE: To avoid exposing chow rats to CAF diet, consider feeding chow cages before CAF cages, or vice versa. Gloves should be changed between handling rats from different diet groups.

3.3. Top up chow, water and sucrose bottles as necessary. Doing this after other procedures is more efficient as boxes are heavier after topping up food and water.

NOTE: CAF boxes soil more rapidly and need to be changed more often to avoid stress induced by exposure to wet bedding as well as strong smells. Inspect bedding daily and change as appropriate.

4. Food intake over 24 h

NOTE: Food intake measurements are conducted over a discrete 24-hour period several times per week.

4.1. When commencing the CAF diet, measure body weight and 24 h food intake at least twice per week to monitor the effectiveness of the diet. Begin food intake measurements close to the onset of the dark phase. Aim to present the same set of CAF foods on food intake measurement days, as equating for flavor and energy densities will permit accurate monitoring of changes over time.

NOTE: This may not be possible for studies requiring >2 measurements of food intake per week, as excessive exposures to the same foods may reduce hyperphagia over time.

4.2. CAF and control diet intake measures

4.2.1. Prepare CAF diet and place into a labeled container for each cage, weighing and recording each component. **Figure 1** shows a completed set of CAF foods. **Table 3** consists of an example food intake monitoring sheet.

4.2.2. Refill water, sucrose and chow levels as required.

4.3. For chow (control) cages

4.3.1. Turn water bottles around (spouts up), place the cage on the workspace and remove the lid.

4.3.2. Weigh the rats and then transfer them to a cage with fresh bedding, together with environmental enrichment.

4.3.3. Record weight of water bottles and chow.

4.3.4. Replace the cage lid, return the cage to rack, and then turn the water bottles around. Small signs stating 'FOOD INTAKE' can be added to the cage to notify attendants and researchers not to touch bottles and chow.

4.4. For the CAF cages:

4.4.1. Perform the same steps as in step 4.3. Additionally scatter the pre-weighed container of CAF foods around the cage with fresh bedding before rats are transferred.

4.4.2. Leave rats for a 24 h period with minimal disturbance. Record any unanticipated disruptions (e.g., arrival of new rats to the colony room, or changes in ambient noise).

4.5. Finish food intake measurement

4.5.1. Record weight of water bottles and chow for the **chow cages**, carefully searching the bedding for pieces of chow. Remove the 'food intake' sign from cages once complete.

4.5.2. Prepare fresh CAF diet for CAF cages.

4.5.3. For each **CAF cage**

4.5.3.1. Record weight of water, chow and sucrose.

4.5.3.2. Remove cage lid and environmental enrichment.

4.5.3.3. Carefully remove CAF fragments from bedding and place into separate containers. Remove the largest pieces first and then systematically sift all cage bedding from one end to the other using gentle sweeping motions.

NOTE: Apply the same degree of care to each cage when collecting food. Record where a cage has been particularly messy—in some instances rats will grind down cake and/or biscuit into a fine powder that is difficult to collect. This can artificially inflate food intake measures.

4.5.3.4. Distribute the new CAF diet around the cage, return environmental enrichment, close the cage lid and return it to the rack, removing 'food intake' sign.

4.5.3.5. Record weight of each CAF food to nearest 0.1 g. Subtract food remaining from the initial food weight to obtain total food consumed per cage, and then divide by the number of rats in each box (thus assuming equal intake).

4.5.3.6. Multiply amount consumed (g/rat) by the energy density (kJ/g) of each food provided by the manufacturer.

NOTE: To calculate macronutrient intake we use assume energy densities of 16.7 kJ/g for carbohydrates and protein, and 37 kJ/g for fat.

REPRESENTATIVE RESULTS:

As shown in **Figure 2A**, CAF diet feeding produces a 2.5-fold increase in energy intake relative to chow controls, based on data from three cohorts of male Sprague Dawley rats, that is consistent over 6 weeks. Other studies have confirmed that this extent of hyperphagia is sustained over 10[21] and 16[22] week experiments. The weight curve (**Figure 2B**) indicates CAF diet feeding leads to a 20% difference in mean body weight compared with control after 3–4 weeks of diet, comparable to the body weight gain consistent with the onset of obesity in humans. At 6 weeks, mean difference in body weight gain between CAF and control groups is 67% (**Figure 2C**) and adiposity, determined by EchoMRI, is approximately doubled (**Figure 2D**). Cafeteria-fed rats typically eat 5–10% of their daily energy as chow (~5 g/rat/day).

Macronutrient intake profiles can be calculated on food intake measurement days using nutritional information from the product manufacturers. We observe consistent macronutrient intakes across sexes and ages, with CAF-fed rats consuming approximately 8% of energy as protein, 34% as fat and 58% as carbohydrate. Our maintenance chow provides 22% protein, 13% fat and 65% carbohydrate. Relative to estimates of macronutrient consumption in human populations (18.3% protein, 44.9% carbohydrate and 30.9% fat in Australia[23]; 15.7% protein, 48.7% carbohydrate and 33.7% fat in the United States of America[24]), our CAF-fed rats consume a lower proportion of energy as protein, a higher proportion as carbohydrate, and a comparable proportion from fat. However, when considering absolute intake, CAF-fed rats overeat all three macronutrients relative to controls (**Figure 3A–C** for males, **Figure 3D–F** for females), indicating that they are not protein deficient. Rather, the macronutrient composition results from the dramatic hyperphagia evoked, which is driven predominantly by excess carbohydrate and fat intake, not usually observed in human subjects who tend to become overweight and develop obesity more gradually.

A recent study we conducted suggests that female rats may be particularly vulnerable to the obesogenic effects of CAF diet. Energy intake was 3.8 times greater in CAF-fed females relative to controls, which was sustained over 6 weeks (**Figure 4A**). A 20% weight difference between groups was observed after only 2 weeks of CAF exposure (**Figure 4B**). After 6 weeks of diet exposure, body weight gain was 12 times greater in CAF rats (**Figure 4C**) and fat mass was doubled (**Figure 4D**) compared with healthy controls. The suggestion of greater susceptibility to diet-induced weight gain in females is supported by an earlier study by Sclafani and Gorman, which showed that a cafeteria diet induced significantly greater weight gain in females than in males[25].

Providing too little of each CAF food may artificially constrain measurements of energy intake. This is most easily addressed by checking that there is some food remaining in the CAF cages 24

h after feeding (when the food is refreshed). **Figure 5** shows how the use of multiple food sets for food intake can lead to substantial shifts in macronutrient intake despite comparable overall energy intake. In this instance, the variability was due primarily to the balance of fat and carbohydrate against a backdrop of consistent protein intake. Analyses of the food sets used indicated that high carbohydrate intakes were observed when a highly-preferred cake was provided; higher fat intakes were observed when this cake was absent.

FIGURE AND TABLE LEGENDS:

Figure 1: Example of cafeteria diet across three days. CAF diet should consist of varied food each day to induce sustained hyperphagia. Preparing CAF foods can be streamlined by placing each cage's food into a designated container. This allows for easy, well-timed delivery into each cage. Day 1: chicken nuggets, beef-flavored dog food, chocolate cream biscuits, jam roll, high-fat purified diet. Day 2: meat pie, chicken nuggets, scotch finger biscuits, caramel mud cake. Day 3: dim sum, chicken-flavored dog food, custard cream biscuits, blueberry cheesecake.

Figure 2: Representative results in multiple male cohorts. CAF diet produces a consistent increase in energy intake (**A**), and body weight (**B**) over 6 weeks in male adult rats. This is accompanied by significant increases in body weight gain (**C**) and fat mass (**D**) when assessed by EchoMRI at 4 weeks of diet. Data are represented as mean ± SEM; n = 48 for individual data; n = 12 for energy intake data (cage as the unit of analysis).

Figure 3: Macronutrient intake in multiple male cohorts. CAF diet exposure increases total intake of carbohydrate (**A**), fat (**B**) and protein (**C**) for male rats. These increases are comparable in female rats for carbohydrate (**D**), fat (**E**) and protein (**F**) intakes. Data are represented as mean ± SEM; n = 12 or n = 4 cages (males and females respectively) averaged over 6 weeks.

Figure 4: Representative results in a female cohort. Cafeteria diet produces a consistent increase in energy intake (**A**) and body weight (**B**) over 6 weeks in female adult rats. After 6 weeks of diet a substantial difference in body weight gain (**C**) and fat mass (**D**) is also observed. Data are represented as mean ± SEM; n = 12 for individual data; n = 4 for energy intake data.

Figure 5: Macronutrient intake over time. Using multiple food sets to assess food intake can lead to differences in macronutrient intake over time given differences in individual preferences in a cohort of male rats. Data are represented as mean ± SEM; n = 4 cages. Note that overall macronutrient intake matches the pattern usually observed; CAF-fed rats consumed 60% energy as carbohydrates, 33% fat and 8% protein, as energy.

Table 1: Example weekly food plan for the cafeteria diet. The CAF diet promotes hyperphagia by providing a variety of palatable foods that are varied daily, as shown in this example weekly food plan. Letters A-E denote unique foods for that food group (for example, Cake A might denote chocolate mud cake and Cake B vanilla sponge). Food intake days, shaded in grey, should be positioned evenly across the week and ideally are kept as consistent as possible. While CAF diet always includes continuous access to healthy chow and water, optional additional daily foods can

include HF or HFHS chows, and 10% sucrose solution.

Table 2: Nutritional information for selected cafeteria food items. This table depicts the nutritional information obtained for several core items in the CAF diet. It is important to ensure that the daily options provide similar macronutrient availabilities, and that rats have access to adequate protein. For each daily set of foods used, it is helpful to calculate the overall energy density and the macronutrient content. The final column contains the recommended starting volume of each food (as energy per rat) for male Sprague Dawley rats at 200 g.

Table 3: Example food intake sheet. The weights of each CAF food item (in grams, per cage) should be carefully recorded on a spreadsheet.

DISCUSSION:

By exposing rats to a variety of highly palatable foods high in fat and sugar, the CAF diet protocol described here provides a reliable and robust model of the so-called 'western diet' eaten by many people. Hyperphagia—assessed as a significant increase in energy intake relative to controls—is observed within the first 24 h of exposure, with statistically significant body weight differences seen within weeks. Thus, CAF is an effective model of diet-induced obesity for rodents.

Several studies have reported that CAF-style diets produce a more exaggerated obesity phenotype than purified HF or HFHS diets. Sampey et al.[26] showed that relative to rats fed a HF diet, CAF exposure led to greater liver and adipose tissue inflammation, poorer glucose tolerance and more insulin resistance. While that study used a lard-based HFD, two other experiments found that CAF diets increased adiposity relative to HFDs based on butter[27] and coconut oil[28]. Similarly, Higa et al. found that while both HF and CAF diets increased visceral fat relative to control diet in mice, only CAF increased food intake and accelerated the onset of hyperglycemia, glucose intolerance and insulin resistance[29]. Another study in mice reported that in addition to more pronounced metabolic effects, CAF exposure increased the incidence of liver and heart pathologies (fibrosis, steatosis and apoptosis measures) relative to a purified HFD[30]. However, recent work has shown that a specifically formulated 'western diet' produced stronger effects on metabolic, adipose and inflammatory measures than a traditional CAF diet[31]. Further work identifying how different types of obesogenic diets affect metabolic outcomes is needed, as there are limited data comparing cafeteria diets with purified diets high in sugar, or high in both sugar and fat.

Most of our work with this model has been with outbred Sprague-Dawley rats. The CAF protocol has been optimized for investigating the metabolic effects[32] of the modern food environment. We have used this model to study the microstructure of feeding across the day[22], intermittent access models of 'bingeing'[33] and in experiments where energy intake is yoked to control levels[34]. More recent studies have examined dietary effects on cognition[20,35] and the gut microbiome[36,37]. Versions of the CAF diet have also been used to study maternal obesity[38] and to explore feeding responses to hypothalamic feeding peptides in obesity[14].

It is important to note that while there is a large body of evidence indicating that the cafeteria

diet induces hyperphagia and obesity in rats and mice, these studies have largely been conducted in outbred Sprague Dawley[13,14,20,39-42] and Wistar[6,26,31,43,44] rats, with relatively few studies performed in Balb/c[17,18] and Swiss[45,46] mice as well as other rodent strains. Therefore it is unknown whether the effects of cafeteria diet reported in the literature will be observed in other strains, especially as there are known strain differences in response to obesogenic diets in both rats[47,48] and mice[49]. Additionally, starting age and weight are also important methodological factors that may modulate the effects of obesogenic diets on metabolic outcomes[25]. Most of our prior work has started rats on CAF diet in early adulthood, including the studies generating the representative data reported here.

Several local factors should also be considered. The food schedule proposed here may need to be modified when setting up CAF diet for use with a different strain or supplier. Local food supplies will determine the specific CAF foods to be used and consumption should be monitored carefully each time a new food is introduced. Nutritional information provided on food packages must be retained and checked over time to ensure that macronutrient calculations are accurate.

Successful application of the CAF model requires careful planning and daily monitoring of cages. Additional time is needed to purchase, thaw and prepare food items daily, and food intake measurement days are labor-intensive. These factors may pose logistical constraints and should be considered when evaluating the model for use. Researchers interested in adapting the CAF model should therefore consider that the reliable hyperphagia and obesity phenotype observed with CAF comes with reduced control over nutrient intake and increased preparation time.

Several limitations of the CAF model are important to consider. Since this model allows rodents to select foods, macronutrient intake cannot be determined for individual rats unless individual housing is employed. While the average macronutrient intake across cohorts is relatively stable, we observe variability in rats' metabolic responses to CAF diet within cohorts, which may relate to differences in individual diet selection. Furthermore, the CAF diet items are not fortified, meaning micronutrient availability may be low. However, our rats always have access to healthy, nutritionally complete chow (which typically comprises 5–7% of energy intake) and are provided with nutritionally complete dog roll as a savory food on 3–4 days per week. It is also important to note that low micronutrient availability is observed in human western-style diets high in fat and sugar, and a high proportion of adults with obesity show micronutrient deficiency[50].

There are also several important caveats to note regarding food intake measurement. As locating all fragments of food is impossible, it is important to ensure that an identical procedure is used for each cage. As food intake is measured on a per-cage basis, we analyze energy intake with cage as the unit of analysis, assuming equal consumption for all rats within. However, because the model is explicitly designed to maximize choice and variety, total energy intake, macronutrient and micronutrient profiles for individual CAF rats are likely to vary. Nonetheless, this provides an opportunity to study individual differences in consumption of, and metabolic response to, the CAF diet. Further studies comparing age-matched male and female rodents across the lifespan will be important to fully characterize sex differences in response to diet. Finally, rodent models cannot—and do not attempt to—recreate the complex range of economic,

psychological and social factors that influence human eating behavior. However, given homologous neural circuitries underlying feeding behavior across mammals, and the similar physiological response to positive energy balance (i.e., in the deposition of fat and altered metabolic function), we believe this model holds value in understanding how poor diets and obesity alter body and brain function.

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DISCLOSURES:

The authors declare no competing interests or disclosures.

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Figure 1

Figure 2

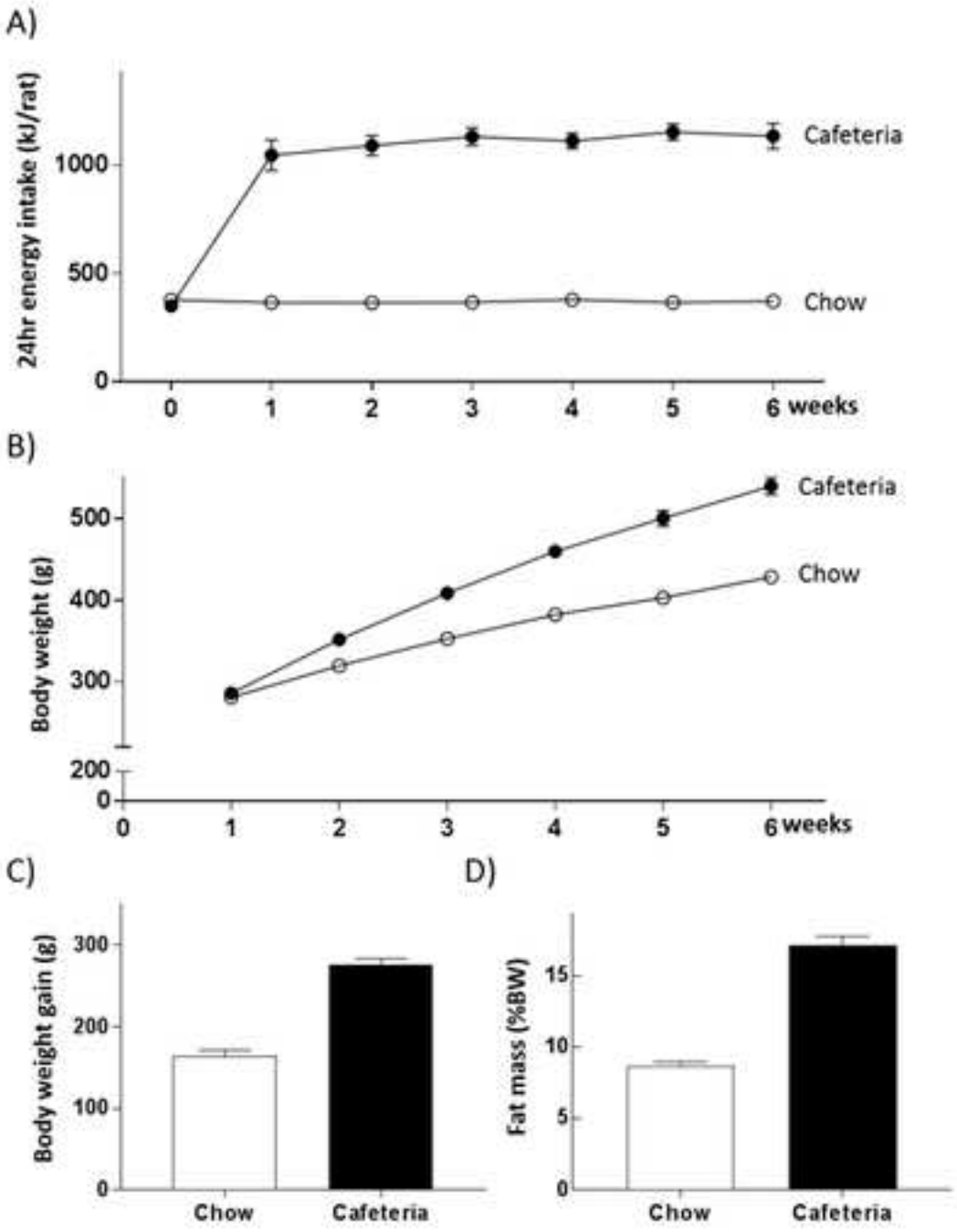

Figure 3

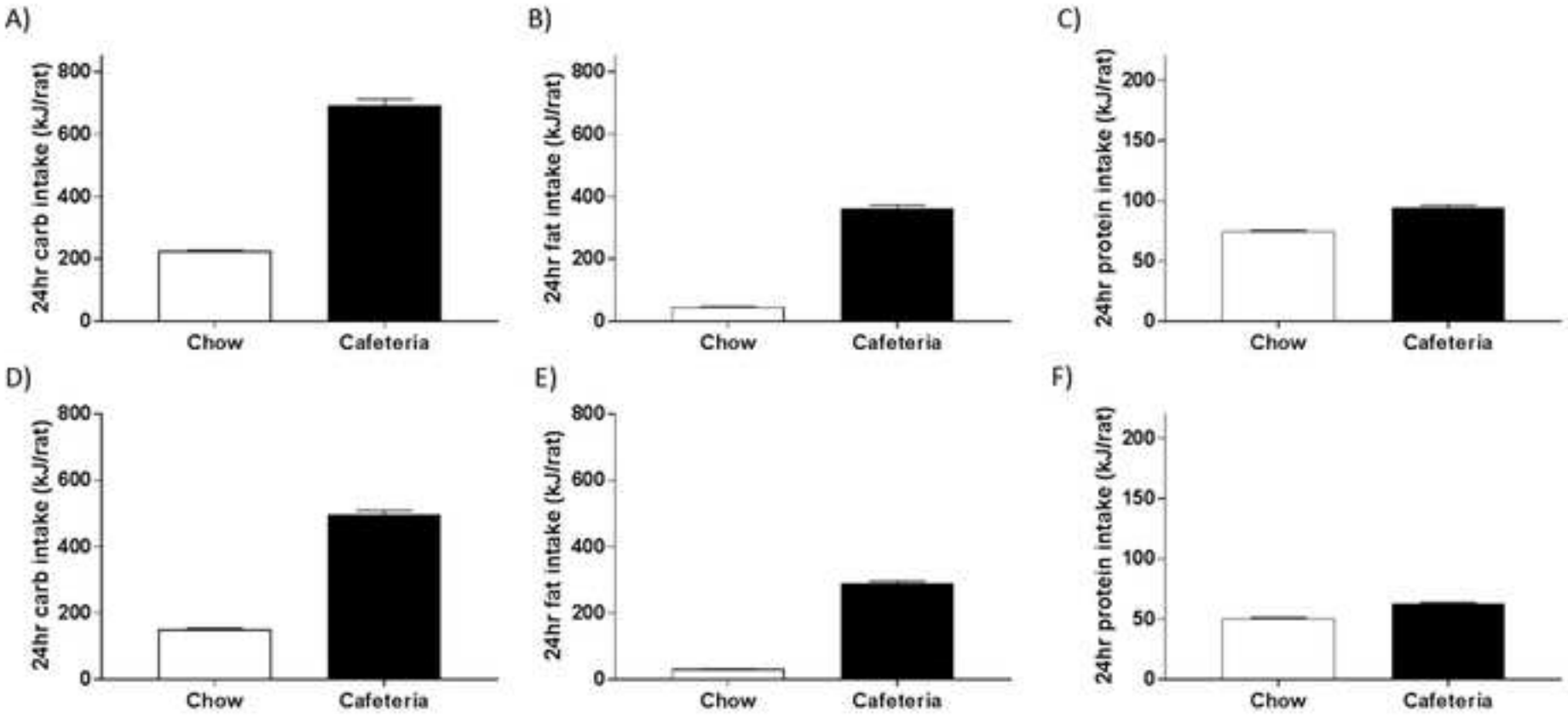

Figure 4

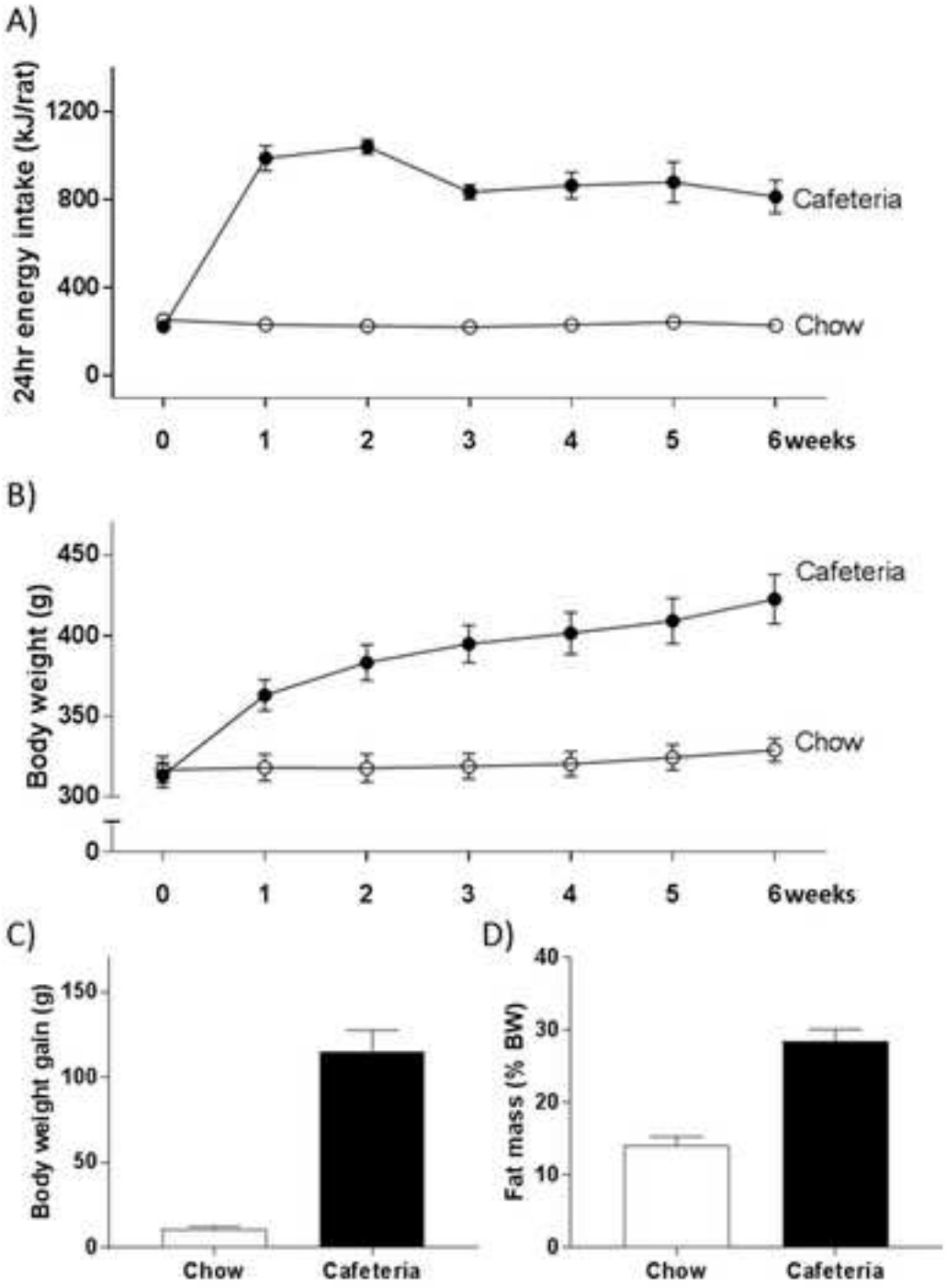

Figure 5

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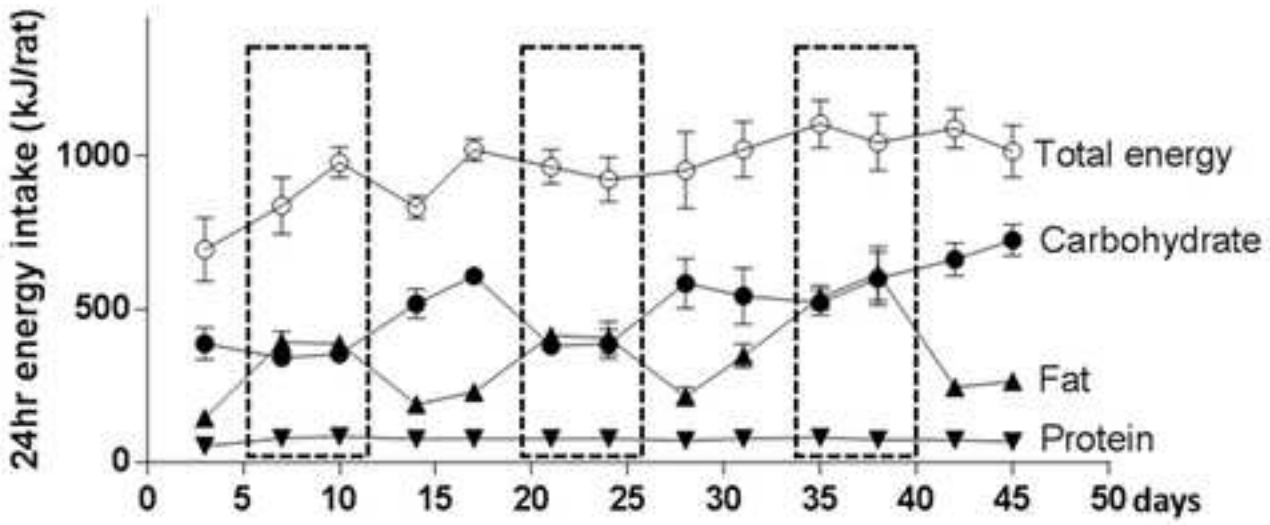

Table 1

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
DAILY FOODS	Healthy chow and potable water					
CAKE	A	D	B	A	C	B
PROTEIN 1	A	C	D	A	C	D
PROTEIN 2	B	B	A	B	B	A
COOKIE	B	A	C	B	D	C
OPTIONAL ADDITIONAL DAILY FOODS	HFHS chow, 10% sucrose solution					

Sunday

E
A
C
A

Table 2

Click here to access/download;Table;Table2_NutritionalInformation.xlsx

PER 100g (derived from manufacturer)

Food	Energy (kJ)	Protein (g)	Total Fat (g)	Saturated Fat (g)	Total Carbohydrate (g)	Sugar (g)
Protein A	830.00	6.00	6.10	3.20	28.90	2.20
Protein B	906.00	7.30	11.10	4.60	21.10	1.80
Cake A	1470.00	4.60	13.30	3.70	52.40	33.10
Cake B	1660.00	4.00	18.40	4.30	53.60	36.30
Cookie A	1920.00	4.30	20.60	12.70	63.20	33.20
Cookie B	2040.00	5.70	21.00	11.20	8.50	4.10

ENERGY PER 1g

Energy (kJ/g)	Protein (kJ)	Total Fat (kJ)	Saturated Fat (kJ)	Total Carbohydrate (kJ)	Sugar (kJ)
8.19	1.02	2.26	1.18	4.91	0.37
8.94	1.24	4.11	1.70	3.59	0.31
14.61	0.78	4.92	1.37	8.91	5.63
16.60	0.68	6.81	1.59	9.11	6.17
19.10	0.73	7.62	4.70	10.74	5.64
10.18	0.97	7.77	4.14	1.45	0.70

Recommended starting value (kJ/rat)
350.00
350.00
200.00
200.00
200.00
200.00

Table 3

Click here to access/download;Table;Table3_ExampleFoodIntakeForm.xlsx

CAGE	WATER (g)		CHOW (g)		Cake A (g)		Protein A (g)	
	IN	OUT	IN	OUT	IN	OUT	IN	OUT
1 (CHOW)					[redacted]			
2 (CAF)								
3 (CHOW)					[redacted]			
4 (CAF)								

Protein B (g)		Cookie B (g)	
IN	OUT	IN	OUT
[redacted]			
[redacted]			

Name of Material/Equipment	Company	Catalog Number
2-5 L plastic bottle		
Chopping board		
Freezer		
Gordon's maintenance rodent chow	Gordon's Specialty Stockfeeds (Australia)	
Large plastic storage boxes		
Large spoon		
Microwave		
Non-serrated knife		
Paper towel		
Plastic containers		
Plastic funnel		
Red light		
Tuna tins		
Weigh container x 3		
Weighing scale		
White sugar		

Comments/Description
For preparing 10% sucrose solution, if applicable
Plastic is advised
For storing CAF foods
Maintenance diet used in our laboratory (14 kJ/g; 65% carb, 13% fat and 22% protein, as energy)
All items above can be stored in containers for easy access
For CAF diet preparation
For CAF diet thawing (when required)
For CAF diet preparation
Important for cleaning work surfaces and the knife during CAF prep
These are for weighing CAF food items on measurement days
For preparing 10% sucrose solution, if applicable
As CAF diet should be refreshed near the onset of the dark phase each day, a red light will assist when working in the dark
For presenting 'wetter' CAF food items. Plastic containers may also be suitable
Separate containers should be used to weigh rats, chow & bottles, and CAF foods
Sensitivity to 0.1g is recommended
For 10% sucrose solution, if applicable

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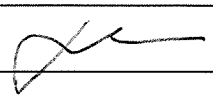
Signature:	
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Dear Editors,

We thank the reviewers for their comprehensive comments, which we believe have improved our manuscript. Below we respond to the questions raised by the reviewers. We are pleased to submit this revision and hope it is now suitable for publication in *JoVE.*

Yours sincerely,

Margaret J. Morris

Michael D. Kendig

Sarah-Jane Leigh

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1) Examples NOT in the imperative: 2.1, 4.1.

***Thank you for drawing our attention to this matter: these and all other incidences of incorrect tense in the protocol have now been corrected*.**

• **Protocol Numbering:** All steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

***Thank you, this has been corrected*.**

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3-page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Notes cannot be filmed and should be excluded from highlighting.
4) Sections 1 and 2 have low filmable content and are best unhighlighted.

***Thank you, sections 1 and 2 are no longer highlighted*.**

• **References:** Please spell out journal names.

All journal names are no longer abbreviated.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, ….

Note, all figures presented in this manuscript are original.

Please note: all line numbers provided below refer to the revised document that includes tracked changes.

Reviewer #1:

Minor Concerns:
L 58. There are inconsistent findings on the ability of flavor variety alone, without variety in nutrient composition, to stimulate overeating and weight gain. Triet et al. 1983 reported that variety alone increased 2-h food intakes but did not measure long-term intake or weight gain. Louis-Sylvestre et al. so-called "isocafeteria diet" varied nutrient composition as well as flavor variety. The authors do not cite the report by Naim et al. (J. Nutr., 1985) that flavor variety alone had little effect on long-term weight gain in rats low-fat or high-fat diets.

Thank you for drawing our attention to the study by Naim and colleagues. We have revised this paragraph (see line 59) as follows:

"Access to variety increases food intake in rats over the short-term[4] and in humans[5] even when foods are matched for palatability and vary only in flavor and olfactory cues[4,6]. However, one study showed that providing energy- and macronutrient-matched purified diets that varied in flavor and texture had no effect on long-term body weight gain in rats[7], suggesting that nutrient composition and distinct post-oral effects of different foods may also contribute to overeating."

L 346. The authors suggest that future studies are needed to compare CAF diet effects in male and female rats. On such study was published by Sclafani & Gorman (Physiol. Behav., 1977) which reported that female rats displayed greater weight gains, relative to chow-controls, than did male rats.

Thank you for drawing our attention to the study by Sclafani & Gorman. We now discuss this study on line 341 as follows:

"The suggestion of greater susceptibility to diet-induced weight gain in females is supported by an earlier study by Sclafani and Gorman, which showed that a cafeteria diet induced significantly greater weight gain in females than in males[28]".

The Sclafani & Gorman study also reported that relative weight gains were greater in older (~6 months of age) than younger (~3 months of age). The present manuscript does not mention age (or starting weight) as a significant factor. However, producing extreme obesity in rats will require longer CAF diet feeding if young rather than older rats are used.

Our studies have typically used young adult rats and so this factor has been less explored in our experience/lab. We now discuss the importance of starting age and weight on lines 450 as follows: "Additionally, starting age and weight are also important methodological factors that may modulate the effects of obesogenic diets on metabolic outcomes[28]. Most of our prior work using the CAF diet has used adult rats, including the studies generating the representative data reported here."

Reviewer #2:

Manuscript Summary:
This is an interesting manuscript, as the focus on rodent diets in many studies is insufficient, though i do believe that the CAF foods could be better matched for protein/cho/lipid contents (nutrient composition databases used for human nutrition studies could be used to do this).

Thank you for this comment. In our representative results section (starting line 308) we now compare macronutrient proportions produced by our CAF model with estimates in people. This shows that relative to humans, our CAF-fed rats consume a lower proportion of energy as protein (8% versus 16-18%) and more as carbohydrate (65% versus 45-49%) but a comparable proportion as fat (34% versus 31-34%).

We would argue that estimates in humans do not distinguish between those eating healthy and unhealthy diets. We aim to model the latter by allowing rats to choose freely from the same variety of palatable foods eaten by people. As all food is self-selected, rats tend to under-eat healthy foods in the presence of palatable unhealthy foods high in sugar and fat. Thus the differences in macronutrient intake appear to develop from rats' preferences for foods higher in sugar and fat content (and somewhat lower in protein).

The revised paragraph (starting line 319) now reads:

"Macronutrient intake profiles can be calculated on food intake measurement days using nutritional information from the product manufacturers. We observe consistent macronutrient intakes across sexes and ages, with CAF-fed rats consuming approximately 8% of energy as protein, 34% as fat and 58% as carbohydrate. Our maintenance chow provides 22% protein, 13% fat and 65% carbohydrate. Relative to estimates of macronutrient consumption in human populations (18.3% protein, 44.9% carbohydrate and 30.9% fat in Australia[23]; 15.7% protein, 48.7% carbohydrate and 33.7% fat in the United States of America[24]), our CAF-fed rats consume a lower proportion of energy as protein, a higher proportion as carbohydrate, and a comparable proportion from fat. However, when considering absolute intake, CAF-fed rats overeat all three macronutrients relative to controls (Figure 3A-C for males, Figure 3D-F for females), indicating that they are not protein deficient. Rather the macronutrient composition results from the dramatic hyperphagia evoked, which is driven predominantly by excess carbohydrate and fat intake, not usually observed in human subjects who tend to develop overweight and obesity more gradually."

Minor Concerns:
Further discussion around how much of the CAF foods should be provided would be of benefit to readers (e.g. grams per g/body weight). Also, the ratio in which they are provided would be beneficial, as figure 1 appears to show different amounts of particular foods provided to different animals.

We do not typically feed our animals based on a 'grams per g/body weight' calculation. Instead, we increase the portion of each food given as rats grow and adjust this based on preferences within each cage (hence the minor variability in amounts shown in Fig 1.). The goal is that rats are not deficient in any nutrient and can freely eat any food (while minimising cost/waste)

Table 2 now includes the approximate starting amount of each food provided (as kJ/rat) for male rats beginning the diet weighing 200g.

Reviewer #3:

Major Concerns:
1. In line 84, the authors argue that "fat and sucrose may not align with human dietary intake." However, is CAF also capable and in what terms? Intake of macronutrients, sugar intake?

As discussed above in response to reviewer 2, we now acknowledge that relative to estimates in humans, our model produces a lower proportion of energy from protein and a higher proportion from carbohydrate. However, macronutrient distribution may vary widely between individuals, and we believe the CAF diet may model the eating patterns of a substantial proportion of the population eating unhealthy diets. Most importantly, CAF diet aims to model the palatability and variety that characterises the selection of foods people have at their disposal.

We have added the following sentence to the Introduction (line 93):

"While CAF diet does not offer the same degree of control over nutrient composition as purified diets, it aims to model the palatability and variety that characterizes food options in most modern societies."

2. There is a lack in protocol on the ethical aspects of the experiment.
As these experiments require daily interaction with the cages (to feed CAF diet) we undertake daily monitoring and observe no changes in rats' activity, sociability or general wellbeing. In 20 years of work with this model we have observed no more incidental deaths in rats on the CAF diet than on control diets. These have tended to occur in longer-term studies when diet exposure exceeds 16 weeks, where occasional cardiovascular events have been observed.

This information is now summarised on line 117 as follows:

' Very few adverse effects have been observed in our short-term studies (i.e. < 10 weeks ad-lib CAF access); there is no evidence of changes to general wellbeing, activity, sociability or anxiety-like behaviour in rats on CAF diet[23]. After longer intervals (>16 weeks) very occasional cardiovascular incidents have been observed in CAF-fed rats.'

3. Is it possible to grind the chow, freeze the palatable foods, turn them into powder and then pelletize the chow with food?

This is an interesting point. Such approaches, which standardise macronutrient proportions between animals, are used by several labs (see "junk-food diet" work by the Ferrario lab, e.g. Robinson et al., 2015, citation below).

The aim of our model is to reproduce elements of overeating in humans by maximising palatability and variety. We believe this has good external validity and, in our experience, yields sustained hyperphagia and a reliable obesity phenotype.

Robinson, M. J., Burghardt, P. R., Patterson, C. M., Nobile, C. W., Akil, H., Watson, S. J., ... & Ferrario, C. R. (2015). Individual differences in cue-induced motivation and striatal systems in rats susceptible to diet-induced obesity. Neuropsychopharmacology, 40(9), 2113.

4. What is the proportion of macronutrient intake in humans? Are the values shown from line 246 comparable to human intake?

See response to reviewer 1; we now compare macronutrient proportions produced by the CAF model to estimates in humans on lines 323-328. The proportion of fat is comparable to estimates from human data, but the CAF model results in a lower % of energy from protein and higher % of energy from carbohydrate, respectively.

5. Do the authors have suggestions on how to deal with the limitations of CAF?

We now discuss the limitations of the model in greater detail on lines 470-480 as follows:

"Several limitations of the CAF model are important to consider. Since this model allows rodents to select foods, macronutrient intake cannot be determined for individual rats unless individual housing is employed. While the average macronutrient intake across cohorts is relatively stable, we observe variability in rats' metabolic responses to CAF diet within cohorts, which may relate to differences in individual diet selection. Furthermore, the CAF diet items are not fortified, meaning micronutrient availability may be low. However, our rats always have access to healthy, nutritionally complete chow (which typically comprises 5-7% of energy intake) and are provided with nutritionally complete dog roll as a savory food on 3-4 days per week. It is also important to note that low micronutrient availability is observed in human western-style diets high in fat and sugar, and a high proportion of adults with obesity show micronutrient deficiency[50]."

Minor Concerns:
1. In the introduction, authors can provide examples of palatable foods.
We now provide examples of palatable foods on lines 55-56 as follows:

"Examples of these foods include cakes, sweet biscuits, and high-fat savory snacks (such as processed meats, cheese and chips)."

2. Line 245. Is there a national table of food composition with the values of these products?
Unfortunately not. Australian law requires food manufacturers to provide estimated energy and macronutrient information (CHO, protein and fat) as well as sodium, fibre and vitamins. We use this to estimate energy and macronutrient intakes.

3. Figure 1. It is more appropriate to show different pictures, for example, a picture for each letter in Table 1.

In response to this comment, we now show food sets for three consecutive days in Fig 1.

4. How was energy intake calculated?

We now describe this process in fuller detail in the protocol on lines 302-306 as follows:

'Subtract food remaining from the initial food weight to obtain total food consumed per cage, and then divide by the number of rats in each cage (thus assuming equal intake). Multiply amount consumed (g/rat) by the energy density (kJ/g) of each food provided by the manufacturer. To calculate macronutrient intake we use assume energy densities of 16.7 kJ/g for carbohydrates and protein, and 37 kJ/g for fat'

5. Is it possible to specify the foods used, such as composition and taste?

As above, we now provide further info on foods on lines 55-56 (and see .

Reviewer #4:

Manuscript Summary:
The authors have described a method for studying weight changes in rats in response to what they describe as increased food variety, compared with standard grain-based chow, and the use of "highly palatable" foods. The manuscript provides a standard operating procedure for those wishing to use the model.

Major Concerns:
The model's value is overstated, and its limitations have been omitted from the manuscript. The authors write, "the CAF diet protocol described here provides a reliable and robust model of the so-called 'western diet' eaten by many people." As a model for human behavior, however, the use of rodents is highly artificial and confounded by a number of variables, some of which the authors have described and attempted to account for.

We now consider the limitations of the model in greater detail on lines 470-480 (see responses to reviewers 2-3 above).

While acknowledging that rodent models cannot capture the complex determinants of feeding in humans, we believe there is value in their use given the neural circuitry underlying feeding, and the physiological changes in response to positive energy balance, are similar across mammalian species. We now discuss this issue on lines 493-499:

'Finally, rodent models cannot – and do not attempt – to recreate the complex range of economic, psychological and social factors that influence human eating behavior. However, given homologous neural circuitries underlying feeding behavior across mammals, and the similar physiological response to positive energy balance (i.e., in the deposition of fat and altered metabolic function), we believe this model holds value in understanding how poor diets and obesity alter body and brain function.'

The authors should describe the rationale for their selection of species. Rats do not have food cultures or fundamental biology that match those of humans, nor does the laboratory environment resemble human

environments. Rather, the species appears to be chosen based on convenience, cost, and ease of manipulation. If true, this should be acknowledged.

Rodents are widely used in biological sciences because data can be generated in a timely manner, with strict control over environmental factors. Rodent studies are also cost effective. As above, rodent research does not aim to recreate the complex social factors that contribute to feeding. This model aims to identify the fundamental role of palatability on overconsumption and the onset of obesity. The response of rats and humans to palatable diets high in fat and sugar is very similar.

Line 51 now states (addition underlined):

"Our understanding of the etiology of obesity has been advanced substantially by work using rodent models, where behavioral, metabolic and molecular effects can be studied across time under controlled conditions <u>where environmental factors can be easily manipulated</u>."

The authors should describe or at least acknowledge the physiological differences in relation to humans that may affect the validity of their model. It would also be useful to note reported differences in rodent strains (for example, it has been reported for many years that rat strains vary in their weight response to varying feeding patterns, as noted for example in Schemmel R, Mickelsen O, Gill JL. Dietary obesity in rats: body weight and body fat accretion in seven strains of rats. J Nutr 1970; 100: 1041-1048.)

Thank you for alerting us to the Schemmel paper; we now acknowledge the importance of strain on lines 444-450.

Although basal metabolic rates vary across mammalian species we wish to reiterate that the process of laying down fat in a scenario of chronic long-term positive energy balance is fundamentally the same.

The authors should also describe the environmental factors that limit extrapolation to humans. The animals are presumably confined and have physical activity patterns that are highly abnormal for rats and inapplicable to most humans. In comparison with rats in the wild, the rats in CAF studies have limited socialization (e.g. four rats per cage), abnormal light-dark schedules, and exposure to noise, smells, and manipulation. Laboratory stresses can be associated with abnormal brain development and behavioral stereotypies and may also alter feeding patterns. For example, in the absence of the ability to explore their environment, rats may eat due to boredom. Similarly, they may stop eating due to stress. While the protocol endeavors to control for some potential confounders by making the CAF and chow-group experiences as similar as possible, it does not adequately discuss the validity of the model in relation to humans.

There are undoubtedly differences between the lives of laboratory and wild rats. However, we are not trying to model a free-living rat, whose lives may be more stressful and unpredictable than our rats. Rather we aim to model how voluntary food intake is affected by food variety and palatability. Our rats are habituated to their colony room and do not exhibit signs of chronic stress, with consistent food intake across our experiments.

It is common for investigators to attempt to make up for the barren existence of cage life with what is euphemistically referred to as "enrichment." In this case, this consists of "a red Perspex box, nesting material and a wooden chew stick." The rats' existence can hardly be termed "enriched." (See for example, Balcombe JP. Laboratory environments and rodents' behavioural needs: a review. Lab Anim. 2006;40(3):217-35.) Instead, the procedures should be described, without the use of the euphemism.

Environmental enrichment is the standard term for material placed in rodents' cages for their wellbeing. As such we would prefer to retain this term, and have ensured that it is fully defined so that readers are not misled.

The authors should discuss humane concerns. To the extent that animals are bred, shipped, confined, stressed, and killed, these humane issues are drawbacks of the method that are of interest and concern to investigators and journals.

We stress that our use of animals has undergone careful review by our institutional ethics committee, who confirmed it conformed with national guidelines for animal welfare. We use rats in order to study the neurological effects of poor diet and obesity and therapeutic interventions. These studies would be unethical if conducted in humans.

It may be relevant to highlight that rodent research has substantially advanced our understanding of the brain circuitry underlying feeding and how these pathways are altered in obesity. All currently-approved pharmacotherapies for obesity are backed by studies in rodents.

The researchers correctly note that their study model is not limited to the study of "variety." In their natural environment, rats would have a much greater variety of foods from which to choose, and that the CAF model does not study this natural variety of foods. Rather, it studies a narrow set of what are often described as "junk foods" drawn from the human marketplace, such as cakes or cookies.

While rats are omnivorous, evidence indicates that the diets of wild rats – whether urban dwelling or in a truly natural environment – are dictated by local availability and seasonal changes (Feng & Himsworth, 2014). In any case, extrapolating to wild rats is not the intention of our model. Rather, we wish to study the effects of long-term consumption of the unhealthy foods eaten by many people.

Feng, A. Y. T., & Himsworth, C. G. (2014). The secret life of the city rat: a review of the ecology of urban Norway and black rats (Rattus norvegicus* and *Rattus rattus*). *Urban Ecosystems, 17,* 149-162.

There is no discussion as to why rodents are used to study a medical condition for which all clinically important issues are currently being investigated in humans. For example, reference 25 describes an attempt to model "yo-yo" dieting in rats, and reference 30 discusses diet effects on the microbiome. Similarly, the authors noted that female rats are especially vulnerable to the obesogenic effects of dietary variety.

The reviewer implies that rodent research is unnecessary to study the sequelae of obesity. While there is indeed ample research on human obesity yielding important findings, there are no studies in which obesity is induced experimentally – this would be highly unethical*. Rather, research compares people with obesity to healthy-weight individuals, or seeks to identify predictors of obesity in longitudinal designs. However, the development of obesity over the lifespan is influenced by an enormous range of factors (genetics, socio-economic status and race, education levels, geographic factors), and can be associated with other adverse outcomes (e.g. weight stigma, mental health issues). Although these variables can be accounted for statistically in human research, research in rodents can ask targeted questions about the neural and physiological effects of obesity without these variables (although subject to other caveats).

****Among the most extreme feeding studies in humans to our knowledge showed that healthy men fed ~6000kcal/day for 1 week gained 3.5kg, became insulin resistant, and exhibited signs of oxidative stress (Boden et al., 2015).***

Boden, G. et al. Excessive caloric intake acutely causes oxidative stress, GLUT4 carbonylation, and insulin resistance in healthy men. Science Translation Medicine, 7(304), 304re7.

Studying these phenomena in humans presents various challenges and economic costs, but has the massive advantage of obviating humane concerns as well as the problems of cross-species extrapolation. While a rat model provides simple, graphic illustrations of the effects of junk food consumption in rodents, the method appears to be mainly a distraction from the kind of research that is needed to address this problem. I hope that the investigators will consider future studies that involve the species of interest—the one affected by the obesity epidemic.

We appreciate the reviewer's thoughtful input. We wish to stress that we are mindful that rodent research is not a panacea for understanding the effects of obesity. It must be integrated carefully with work in humans. Many people overconsume so-called junk foods and gain excess weight across their lifespan, increasing the risk of chronic disease and early mortality. Inducing this in humans is highly questionable, yet solutions are needed to reduce the disease burden of obesity. To do that we need to understand what changes in the body and brain as obesity develops. We believe rodent models such as this one can help work towards this.

Minor Concerns:
None

Reviewer #5:
Major Concerns:
While the protocol is very thorough, the video format of JOVE might not best represent this methodology.

We believe this protocol involves careful planning and can be time-consuming. By providing a detailed set of instructions we hope that this protocol will facilitate reproducibility, complemented by the video.

The current data do not utilize a traditional HFD to directly compare the advantage of the novel CAF to chow fed rodents.

As noted on lines 420-434, several past studies have directly compared CAF to purified high-fat diets (Sampey et al., Higa et al.; Zeeni et al., Buyukdere et al., Oliva et al.), with each reporting more severe metabolic effects produced by CAF.

I would consider reorganizing the introduction. It is well known that obesity is bad and getting worse. A quicker transition to the history and previous work with CAF would allow for a more comprehensive description of this and comparisons to traditional HFD.

We have curtailed the opening paragraph but believe it is important to place the method in context.

Similarly, the discussion could expand on the relative efficacy of CAF diet to induce not only obesity, but also related comorbidities such as NAFLD, T2D, hyperlipiemia, hyperleptinemia, etc compared to refined ingredient HF or HFHS diets. As currently written, most of the discussion describes notes relevant to the feeding of rodents that has already been discussed. More information should be provided to expand on the metabolic differences between the CAF and traditional HFDS beyond obesity and hyperphagia.

Thank you for this suggestion. We had discussed the differences between CAF and purified HFD in the introduction, and have now moved this section to the discussion, and expanded to include three other studies where the two diets were compared directly on metabolic outcomes. The revised paragraph (lines 420-434) is as follows:

"Several studies have reported that CAF-style diets produce a more exaggerated obesity phenotype than purified HF or HFHS diets. Sampey et al.[26] showed that relative to rats fed a HF diet, CAF exposure led to greater liver and adipose tissue inflammation, poorer glucose tolerance and more insulin resistance. While that study used a lard-based HFD, two other experiments found that CAF diets increased adiposity relative to HFDs based on butter[27] and coconut oil[28]. Similarly, Higa et al. found that while both HF and CAF diets increased visceral fat relative to control diet in mice, only CAF increased food intake and accelerated the onset of hyperglycemia, glucose intolerance and insulin resistance[29]. Another study in mice reported that in addition to more pronounced metabolic effects, CAF exposure increased the incidence of liver and heart pathologies (fibrosis, steatosis and apoptosis measures) relative to a purified HFD [30]. However, recent work has shown that a specifically formulated 'western diet' produced stronger effects on metabolic, adipose and inflammatory measures than a traditional CAF diet[31]. Further work identifying how different types of obesogenic diets affect metabolic outcomes is needed, as there is limited data comparing cafeteria diets with purified diets high in sugar or high in both sugar and fat."

Line 108 - how long is the acclimatization/handling period? Is there a specific phenotype the investigator should be monitoring to assess when sufficient handling has occurred?

As specified on line 126, we typically acclimate rats to the facility for 5-7 days and handle them daily beginning 24-h after arrival. There is no specific phenotype or criteria; rather we observe that rats cease urinating and defecating during handling; do not freeze and explore the handler; and generally appear relaxed.

Line 115, Group Allocation - rats (especially male rats) form social hierarchies in which one rat displays social dominance over the other rats. One way in which this dominance can manifest is in the control of access to food, which might be expected to be more pronounced when highly palatable foods such as those used in the CAF diet are available. Since the non-dominant rats can still eat regular chow, this can lead to a stratification within a cage wherein the dominant rat(s) consume more of the CAF food while the others eat chow. Have the investigators - who have considerable experience using this diet - ever observed this behavior or significant intra-cage variability? If so, how have they handled this?

This is an interesting and important point; thank you for raising it. We aim to prevent dominance interacting with the diet's effect by distributing food evenly around the cage. While we weight-match cages at the beginning of the diet, on occasion substantial within-cage variability develops. We believe in most cases this is due to genetic susceptibility. Importantly, we do not observe any greater incidence of fighting or differential social behaviour in our CAF cages. We now explain this on lines 141-144 as follows:

"*Note: Rodents form social hierarchies when group-housed (especially males). The effects of social stress are partially controlled for by ensuring rats are hosed with others of a similar body weight (to reduce bullying within a cage). Additionally, cafeteria diet should be evenly distributed around the cage so that all rats have access to the diet."*

Line 137, Wet Foods - if a tuna tin or similar container is used in the CAF cages, are the chow rats given empty containers (or similar containers with chow food in them)?

Yes we do: this is written on line 168.

Line 146, Sucrose Solution - when sucrose water is offered, is this in place of plain water or in addition to?

Sucrose solution is always offered in addition to plain potable water so that all intake is voluntary. A note has been added (line 185) to reiterate this.

The male and female body weight gain/change should be consistent. Males are shown as body weight gain, while females show raw body weight values. Further, there is no macronutrient data given for females and no sex is identified in Figure 5.

We now show raw weight values for males and present macronutrient data for females in Figure 3. The legend for Figure 5 now clarifies that this is data for males.

Also in Figure 5 - if the variation in the pattern of fat/carbohydrate intake is due to differences in the macronutrient composition of the foods presented that day (e.g., high-fat food on Day 10 vs lower-fat foods on Day 15), this should be designated on the plot or in the figure legend.

Figure 5 is intended to show suboptimally 'matched' foods across food intake measures. We now state this in the figure legend.

Minor Concerns:
Abstract, line 36: The labor intensive aspect of this protocol seems out of place in the abstract. I would remove this and reorganize the rest of the information in the sentence with the previous sentence.

We have removed the phrase, 'while labor-intensive' from the abstract.

Page 1, line 53: remove 'herein' from the parenthetical.

Now removed, thank you.

Page 1, line 66: keep tense consistent (eg. "overcomes sensory-specific satiety, or the decreased desire to eat…")

We prefer to retain the present tense for this statement as it defines a phenomenon shown across many studies, sensory-specific satiety, rather than reporting a result from a previous paper.

Page 1, line 85: remove "see" from the parenthetical.

Now removed, thank you.

Page 1-2, lines 88-91: Too many sentences with "another study". Consider varying sentence organization.

We have revised this section to introduce some variety in sentence structure.

Page 2, lines 95-97: This warning makes it sound like the CAF is a bad idea. Could be removed?

Thank you; we prefer to retain this but have shifted to the discussion.

Page 2, lines 110 and 113: Avoid excessive adverb use (eg particularly and especially)

Thank you; both removed.

Page 2, line 124: Replace 'cakes' with 'sweet items' to retain usage consistency.
Thank you, this is now amended.

Page 4, line 181: Condense to "…procedures is more efficient as boxes are…"

Thank you, this is now amended.

Page 4, line 213: Condense to "…24-h period with minimal disturbance."
Thank you, this is now amended.

Page 7 line 318: This is not 'against' an outbred background. Do you mean 'in' an outbred rat strain? This is also the first time 'outbred' is mentioned - although more detail is given in the next paragraph. This should be reorganized; if the authors wish to discuss genetic background, they should include information regarding the validity of this approach in inbred models, particularly mice, which are widely used in metabolic and obesity research.

Thank you. We do mean in an outbred strain and clarify that our Sprague-Dawley rats are outbred. We have added some discussion on evidence regarding different strains (lines 445-451).