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Practice Guideline

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Chowing down: diet considerations in rodent models of metabolic disease

Kevin C. Klatt[1], Kevin Bass[2], John R. Speakman[3], Kevin D. Hall[4,*]

[1]Department of Nutritional Sciences and Toxicology, University of California Berkeley, Berkeley, CA 94720, United States
[2]Garrison Institute of Aging, Texas Tech University Health Science Center, Lubbock, TX 79430, United States
[3]Center for Energy Metabolism and Reproduction, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, Guangdong 518055, China
[4]National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, United States

*Corresponding author. National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, United States. E-mail: kevinh@niddk.nih.gov

Abstract

Diet plays a substantial role in the etiology, progression, and treatment of chronic disease and is best considered as a multifaceted set of modifiable input variables with pleiotropic effects on a variety of biological pathways spanning multiple organ systems. This brief review discusses key issues related to the design and conduct of diet interventions in rodent models of metabolic disease and their implications for interpreting experiments. We also make specific recommendations to improve rodent diet studies to help better understand the role of diet on metabolic physiology and thereby improve our understanding of metabolic disease.

Keywords: diet composition; nutrition; chow; study design; recommendations

Introduction

The 1926 Nobel Prize in Physiology and Medicine was awarded to Johannes Fibiger for his discovery that stomach cancer could be caused by a parasitic roundworm infection in rats and mice. Unfortunately, Fibiger's rodent diets had insufficient vitamin A, and it was later found that roundworm infection did not produce stomach cancer when this dietary deficiency was removed [1]. Fibiger's Nobel Prize was described as "one of the biggest blunders made by the Karolinska Institute" [2] and served as a warning that nutrition can seriously confound the interpretation of mechanistic studies in experimental rodents.

To address concerns about potential confounding and reproducibility that may result from deficient or highly variable diet compositions across laboratories, national efforts were launched to standardize nutrient requirements of laboratory rodents [3]. Nutrient requirement estimates for the mouse, last published by the National Research Council in 1995, have been established for total energy and its contributing macronutrients, protein and amino acids, minerals, and vitamins. The indicator of requirement is commonly set as the dietary intake necessary to facilitate growth, reproduction, lactation, and/or the maintenance of adult health. Prior to the 1970s, the nutrient requirements of the laboratory rodents were met using closed diet formulations (i.e. proprietary ingredient composition) that relied primarily on natural ingredients (e.g. minimally refined whole grain and fish meals). In 1974, Knapka *et al*. developed the first open-formula diet (i.e. known ingredient composition) termed the NIH-07 formulation, to serve as a cereal grain-based, non-purified standard reference diet that met the 1962 National Research Council recommended nutrient concentrations for mice [4]. This formulation provided the foundation for the various, yet increasingly dated, rodent diet formulations employed today, the most common of which being the AIN93 series (Supplementary Table S1) [5–7].

Efforts to standardize diet formulations were ultimately derived from efforts to provide the essential nutrients, while minimizing the variability diet might introduce. Such an approach engenders a view of laboratory animal nutrition as a factor to be fixed, rather than a key aspect of experimental design to be considered in each investigation. However, diet is now increasingly recognized as much more than a nuisance variable. Rather, diet is best conceptualized as a multifaceted set of modifiable input variables with pleiotropic effects on a variety of biological pathways spanning multiple organ systems. Diet is a key modulator of metabolic physiology and plays a substantial role in influencing what we might identify as "normal" physiology, as well as the etiology, progression, and treatment of chronic disease [8, 9]. The broad, complex physiological effects of diet interventions present both unique opportunities and challenges for the design of rigorous studies and their interpretation. Herein, we discuss key issues related to diet interventions in rodent models of metabolic disease and their implications for interpreting experiments. We also make specific recommendations to improve rodent diet studies to more fully leverage the powerful effects of diet on metabolic physiology to improve our understanding of metabolic disease.

Compared to what? Diet design hierarchy

Unlike other experimental variables, diet interventions have no placebo or other obvious control. Thus, causal inferences must be

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drawn from comparison diets that can differ in at least one, and often many variables (i.e. diet ingredients). Diet formulations are based on refined vs. "natural" (i.e. unrefined) ingredients, which determines the capacity of investigators to manipulate diet composition in a controlled manner. Natural diet ingredients contain multiple nutrients and bioactive compounds in variable quantities thereby limiting the ability of investigators to modify components individually. Within vivariums, animals are commonly fed "chows", an unstandardized term that most often refers to cereal grain- and legume-based formulations supplemented with refined ingredients that facilitate adequate nourishment of rodent colonies in a readily affordable manner.

Unlike chows, purified diets are formulated using refined ingredients (e.g. sucrose, corn starch, casein, refined oils, cellulose fiber, and micronutrient mixes), enabling precise manipulation of individual dietary components. However, this increased experimental control comes at significant financial cost and the exclusion of many dietary bioactive compounds that are otherwise present in natural diets. Ultimately, the appropriate diet formulation depends on the level of specificity desired when causally attributing observed effects to specific diet differences and identifying the underlying biological mechanisms. Below, we highlight these diet design challenges as they apply to both refined and natural diet formulations.

Dietary pattern designs

Often, experiments compare the effects of two diets that differ in numerous components (Fig. 1a; Supplementary Fig. S1). In such "dietary pattern designs", causality with respect to measured phenotypes can only be attributed to some undetermined combination of diet differences. Common dietary pattern designs include the comparison of low-fat control diets to diet-induced disease models (e.g. Western or Atherogenic diets), wherein numerous ingredients and nutrients differ between formulations. Rigorous dietary pattern studies utilize purified ingredients, limiting the number of ingredient differences between the intervention and control diets and ensuring that such differences are quantifiable/known.

Unfortunately, it is common to find dietary pattern studies using "low fat" control groups consuming natural foodstuffs (i.e. "chows") that are inappropriately compared with experimental groups consuming purified diets (Fig. 2; Supplementary data). Such experimental designs (corresponding to a "dietary pattern design"; Fig. 1a) [10, 11] maximize formulation differences and increase the potential for confounding. This common approach is problematic for causal inference, as chows lack standardization across all nutritionally relevant parameters [12]. Furthermore, rodent chow varies from lot to lot within the same product line with respect to major bioactive components, including but not limited to native factors such as fibers and plant secondary metabolites (e.g. polyphenols, carotenoids, phytate), as well as contaminants and processing-related bioactives such as heavy metals, pesticides, mycotoxins, advanced glycation end products, and lipopolysaccharide. Differences are further exacerbated by the need to sterilize chows (e.g. autoclave, irradiate, etc.), introducing further compositional variability of bioactive food components [13]. Importantly, much of the variability within chows goes routinely unmeasured in standard compositional analyses, and many such factors can be profound determinants of physiology and metabolism. For example, recent investigations have detailed large variability across common vivarium chows in their Fermentable Oligo-, Di-, Monosaccharides And Polyols contents, a class of microbiota-accessible carbohydrates that modify microbiome composition and cecal metabolite concentrations [12]. Collectively, batch-to-batch variability potentially undermines reproducibility even within identical commercial diet lines [14–18].

As an example of how using chow controls can lead to misleading results, a series of high-profile studies on so-called obesogenic microbiota attributed increased obesity in groups of mice fed purified high-fat diets (HFDs) to differences in microbiota composition compared to mice fed unrefined chow diets [19–21]. Unfortunately, it was later demonstrated that mice fed purified low-fat diets, like their purified HFD counterparts, also developed "obesogenic microbiota" while nonetheless not gaining excess body fat, suggesting that the observed microbiota differences may have been due to the refined nature of the HFD rather than causing or being caused by obesity *per se* [22]. Given the widespread inappropriate use of chow diets as controls for purified diets, this cautionary tale raises disconcerting questions about the number of potentially misinterpreted and misleading studies.

While researchers commonly employ "dietary pattern designs" to induce a phenotype (e.g. obesity and insulin resistance), attribution of specific dietary factors causing the phenotype is complicated by the numerous differences between experimental and control diets. The feeding of controls with as few composition differences as possible, can improve the likelihood of identifying specific diet factors causing the phenotype. Alternatively, using a variety of different diets to induce similar phenotypes can limit the potential that observed physiological relationships are not merely secondary to a particular choice of diet composition. For example, researchers intending to study the impact of obesity and insulin resistance on various organ systems can do so through the use of separate HFDs rich in either monounsaturated or polyunsaturated fatty acids (compared to refined low-fat controls), reducing the likelihood that observed phenotypes are secondary to alterations in specific dietary fatty acids and their impact on tissue lipid composition and related signaling (e.g. eicosanoids) as opposed to the effects of obesity and insulin resistance *per se*.

Diet substitution designs

To narrow the scope of diet differences that may be responsible for any observed effects, "diet substitution designs" specify that any diet component with substantial mass or energy content that is modified in one arm of the study must also have a corresponding component of the same mass or energy modified in the comparison diet (Fig. 1b). This avoids differential concentration or dilution of dietary components when a single component is added or subtracted from the formulation and avoids mismatching all other dietary components per unit mass or energy. Researchers often employ dietary pattern or diet substitution designs to target a specific metabolic mechanism, but two-armed designs are inherently confounded when it comes to inferring the causal contributions to specific dietary factors. Even in the simplest diet substitution design, pairwise comparisons of diets always differ in at least two variables, rendering two-arm diet investigations insufficient for causal inference about a particular diet component to a study outcome. Outcome differences may be due to the addition of an individual component, the removal of the component that was replaced, or some combination. In other words, diet substitution effects require that investigators consider potential effect modification due to the *substitution* component when making causal attributions.

For example, studies that investigate effects of replacing, gram-for-gram, individual essential amino acids, such as methionine or

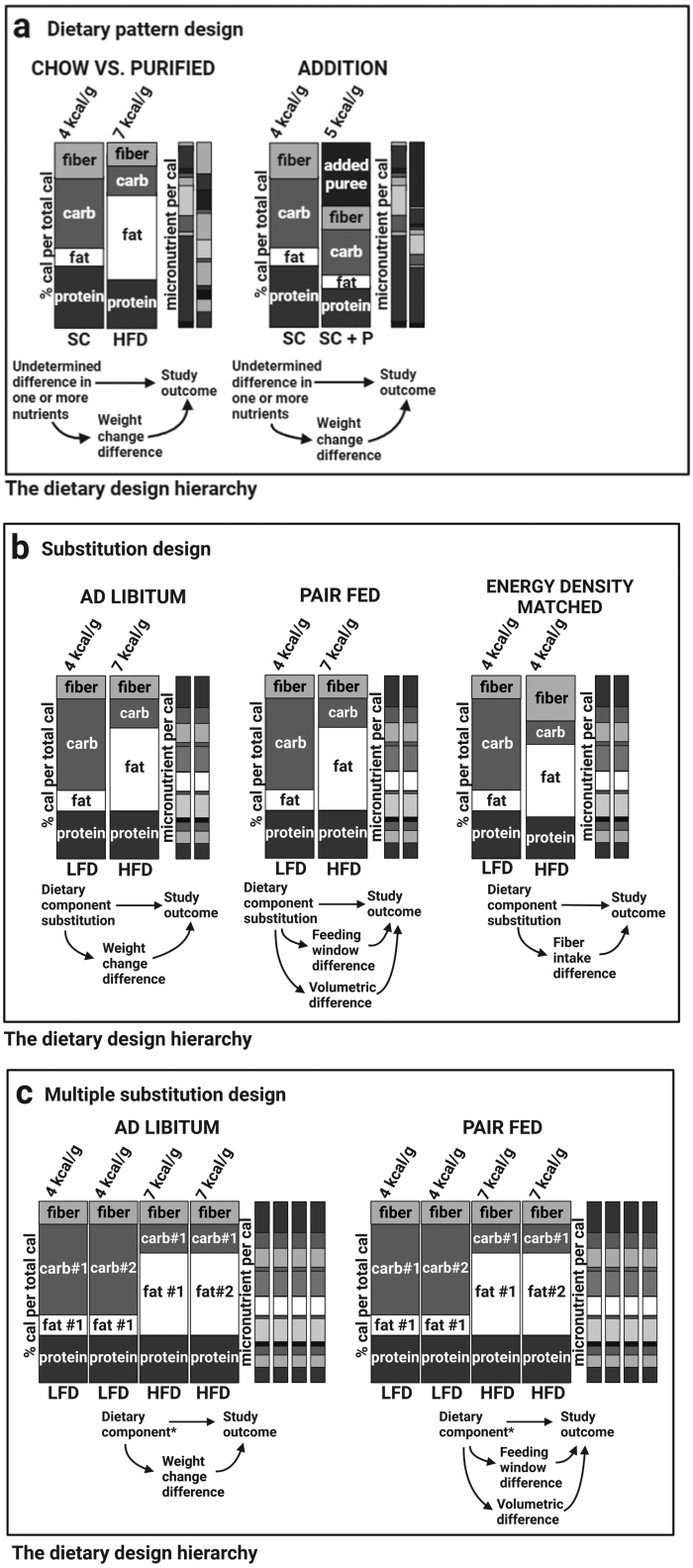

Figure 1 Diet design hierarchy. (a) Dietary pattern design. Chow vs. purified design. All nutrients between standard chow (SC) and HFD are mismatched. An undetermined component or components within the overall dietary pattern are responsible for the study outcome. No further causal inference is possible on the basis of the experimental diets alone. Addition design. All nutrients between SC and SC + puree (SC + P) are mismatched. An undetermined component or components within the overall dietary pattern are responsible for the study outcome. No further causal inference is possible on the basis of the experimental diets alone. (b) Substitution design. *Ad libitum* design. Matched protein, micronutrients, and fiber content. Mismatched energy density may cause excess calorie intake in the HFD group compared to low-fat diet (LFD) group. Pair-fed. Matched protein, micronutrients, and fiber content. Mismatched energy density may cause excess calorie intake in the HFD group compared to LFD group; pair feeding can equalize for differences in energy intake. However, pair feeding may introduce differences in feeding times, lengths of feeding windows, or volumetric differences with physiological import. Energy density matched. Matched protein, micronutrients, and energy density. However, mismatches in fiber content may have independent physiological import. (c) Multiple substitution design. An extension of the substitution design, a multiple substitution design creates relative rankings of all nutrients of interest with respect to an outcome or outcomes of interest.

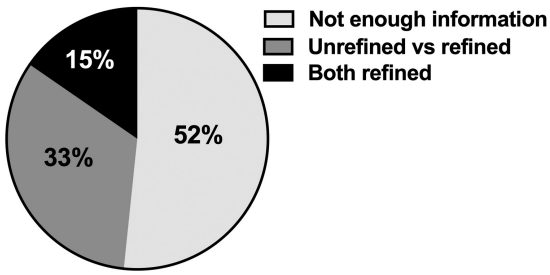

Figure 2 The prevalence of unrefined comparators for refined diets across top biomedical research journals. The inappropriate use of unrefined dietary formulations as comparators for refined dietary formulations, exhibiting numerous quantified and unquantified compositional differences, is highly prevalent. To estimate the prevalence, all papers published between 2019 and 2020 in the journals *Cell Metabolism*, *Diabetes*, *Journal of Clinical Investigation*, *Nature*, and *Nature Medicine* found in PubMed via the search terms "high-fat mouse" and including a diet-versus-diet comparison in at least two groups of mice, were included for analysis, for a total of 91 papers (Supplementary data). Fourteen (15%) papers compared refined to refined diets; 30 (33%) papers compared unrefined to refined diets; and 47 (52%) papers did not provide enough information. These data show that, consistent with previous reports [10, 11], inappropriate dietary design and reporting remains the norm, even in papers published in the highest-impact journals.

leucine, with individual non-essential amino acids [23–26]. Such nutrient substitution otherwise maintains the same protein, energy, and nutrient composition of each diet and isolates the change in diet to a single amino acid, isolating the specificity of causal inference to that single substitution. This example highlights an ideal case: a precise, purified amino acid substitution. However, in less ideal circumstances, diet substitution designs can still introduce substantial uncontrolled confounding. This can occur when substituting ingredients with multiple components, e.g. substituting oils containing multiple different fatty acids as well as phytosterol and vitamin E contents, in variable quantities in each oil. Such a substitution confounds causal inferences with respect to the target fatty acid substitution. Substitutions may also introduce substantial confounding when they result in divergent total food intake in terms of energy or mass, commonly observed when formulation changes modify palatability or energy density.

For example, isocaloric exchange between dietary fat (~9 kcal/g) and carbohydrate (4 kcal/g) results in matching all other diet components on a per energy basis but not per unit mass. As many other elements of a diet are based on the formulation weight (e.g. energy density of the diet; vitamins, minerals, and fiber commonly added at a % w/w), such substitutions, as commonly employed in "High Fat Diets", can induce differences in intake beyond the isocaloric exchange that may have independent effects on outcomes of interest. In instances where caloric intake is expected to be the same across groups despite changes in energy density, ingredients in the formula can be added on a weight per total kcal of the formulation basis, as opposed to % w/w, to avoid mismatched intakes. Food intakes often diverge when energy densities have been altered, and thus, some have proposed the addition of non-caloric ingredients to the formulation to normalize energy densities (e.g. the addition of cellulose); however, this introduces another mismatch by diluting or concentrating other diet components per unit mass and assumes a completely inert effect of the added ingredient. Attempts to match food intake between groups can induce other behavior differences that also need to be considered when interpreting outcomes. Finally, despite this tremendous advantage over the dietary pattern design, even a perfectly executed two-armed diet substitution study without the above problems cannot disentangle the effects of the component that was restricted from the effects of the component that was added to replace it; this limitation is inherent to the substitution design and cannot be overcome so long as the design is used.

To overcome the limits to causal inference inherent to two-arm designs, a "multiple diet substitution design" is needed by adding additional comparator groups, each making a different substitution, thereby producing a ranking of the relative impact of each diet component on the outcome of interest. In other words, the physiological effects of a single diet component may be isolated by making multiple substitutions and examining whether the outcomes remain robust and examine effect modifications due to the substituted components (Fig. 1c). For example, a methionine restriction study could compare multiple diets that are restricted in methionine but differ in their replacement of amino acids and thereby determine whether the observed effects are robust for all such substitutions. If dose-response relationships are also of interest, the necessary studies become increasingly more complex to instill confidence about the relative impact of individual diet components. Unfortunately, while essential for inferring causal contributions of individual diet components, such multi-arm studies are expensive and burdensome, and involve a large number of comparisons.

Embracing diet complexity as a probe of physiology

Conceptualizations such as the "Geometric Framework for Nutrition" [27], have encouraged the field to move away from attempts to modify a single dose of a dietary variable and to embrace nutrient substitution matrices that consider potential interactions across dose-response ranges in order to advance our understanding of nutritional phenotypes. This involves conducting large studies using a wide variety of chemically defined (i.e. purified) diets. Detailed phenotyping using physiological characterizations, isotope tracing, multi-omics technologies, and other quantitative methods, together with genetic and pharmaceutical perturbations along proposed causal pathways can be used to strengthen causal attributions and elucidate diet-induced phenotypes.

Several investigators have used such a multivariable diet approach to investigate important questions regarding macronutrients and caloric intake, adiposity, reproductive function, and longevity [28–30]. For example, to address the effects of dietary macronutrient distribution on energy intake, expenditure, and adiposity, Hu *et al.* [31] employed 29 diets across various permutations of total fat (8.3%–80%), carbohydrate (10%–80%; 5%–30% as solid sucrose), and protein (5%–30%), maintaining the fatty acid composition of the diets in mice. Across all dietary permutations, dietary fat, provided as a single mixed oil source to match the relative saturated, monounsaturated, and polyunsaturated fatty acid composition of the Western diet, was the key driver of energy intake and adiposity through 50%–60% intake, independent of varying protein content and carbohydrate level and types (corn starch, maltodextrin, and sucrose). Using the same 29-diet matrix, Hu *et al.* [32] failed to detect any further impacts of diet composition on glucose tolerance once the effects of adiposity were controlled. This large undertaking highlights the strength of designs that go beyond the commonly employed two-arm design. However, despite using 29 different diets, additional factors, such as variation in fiber and micronutrient intakes, as well as fat

composition and energy density, remained unexplored in these studies, limiting sole causal attribution to total fat intake *per se*.

As researchers embrace diet complexity, it becomes apparent that one single background diet composition is not likely to meet the needs of a research community and existing formulations may be suboptimal for understanding the complexity of both physiology and disease. There is a great need for renewed interest in modifying diet as an experimental variable in biomedical research to facilitate discovery that includes commonly modified ingredients (e.g. sources of macronutrients) as well as the vast array of bioactive compounds found in natural foodstuffs currently omitted from purified formulations.

Design and measurement challenges

Assessment of intake

Assessing the validity of any test of a diet-related hypothesis requires the assessment of intake, though this is a challenging task. Individual measurements of food intake in group-housed animals are not possible unless sophisticated hopper systems enable the intake of chipped individuals to be monitored. In the absence of such a system, reporting food intake of the group on a per animal basis by dividing by the number of animals in the cage can be misleading. However, it cannot be assumed that animals that are group-housed consume equal amounts of food.

Furthermore, the standard procedure to measure food intake is by the difference in weight of food in the hopper over a given period. However, this fails to account for food losses, as not everything that leaves the hopper is consumed and bits of food in the bedding must be accounted for to get an accurate measure of intake. The extent of food loss depends on the hardness of the food with softer foods being more easily fragmented and lost [33]. In addition to wastage, all ingested food does not get absorbed. Accurate estimates of total energy absorption, therefore, need to account for losses in feces (i.e. assimilation efficiency). This is almost never done (but see for example in [31]), yet differences in absorption efficiency between individuals can explain relatively large changes in adiposity over periods of 3–4 weeks [34, 35]. This is a neglected area in rodent dietary studies but has significant implications for experimental interpretation with respect to the relative energy intake between different diets, coprophagy, etc.

With respect to the latter, it is important to consider that the nutritional status of rodents is influenced by not only laboratory diet selection but also by consumption of feces ("coprophagy") and bedding. Coprophagy is a source of several micronutrients (e.g. vitamins B and K) and other postbiotic bioactives (e.g. short chain fatty acids, plant secondary metabolites) due to microbial metabolism ("postbiotics") [36–38]. The impact of such factors on rodent nutrition and physiology are increasingly considered in the era of microbiome investigations [39, 40]. For example, choice of bedding has been shown to modify microbiome composition, metabolic outcomes, and fecal energy content [41, 42]. Despite this, few investigators have tested the interaction between dietary interventions and the presence or absence of coprophagy [40].

When food intakes are altered by diet composition, pair feeding may be employed to separate the effects of the diet itself from its confounding secondary effects on intake and body composition. In a pair feeding protocol, the mice exposed to the test diet are only supplied with sufficient food to match the calorie intake of the control group. This can be done either with or without adjusting for potential differences in assimilation efficiency between the groups. Pair feeding, however, can also generate confounding effects through altering the timing of intake, depending on the exact protocol. For example, if the pair-fed group is given their ration around the time of lights out (~ZT12), the animals may consume the entire ration over a short period, thereby creating a prolonged fasting duration relative to the *ad libitum* control animals. As a result of the differing fasting periods, different metabolic outcomes may result despite the same calorie intake throughout the day [43]. Moreover, in such a scenario, animals sacrificed in mid-afternoon will not only differ in their diet composition but also how long since they last fed.

Analogous to pair feeding, many time-restricted feeding protocols match total intakes but introduce confounding via altering the interval between feedings/degree of fasting as well as the timing of intake within the circadian cycle. This type of effect can be removed by using automated feeders that distribute the food to the pair-fed animal by temporally yoking its total intake and the time-matching feedings to that of a specific control. Collectively, total intake, duration of feeding and fasting, as well as intake patterns in relation to the circadian cycle should all be considered as potential factors that may contribute to the impact of diet composition on measured outcomes.

Randomization and sample size

While it is commonplace to power studies based on individual rodent number, group-housing introduces non-independence of individual-level phenotypic data (e.g. body weight, gene expression, etc.); this non-independence presents a challenge for nearly all experimental variables, but is exacerbated in the case of diet, an intervention delivered at the level of the cage. Thus, sample size and statistical power calculations need to consider diet interventions delivered at the level of the cage as cluster-randomized interventions. The analyses of such data from group-housed animals should be handled by including the cage as a random effect in a mixed effect model. Unfortunately, few investigators report designing and analyzing dietary intervention studies in group-housed animals appropriately, rarely providing the number of clusters (i.e. number of cages) and sample size per cluster (i.e. number of animals housed per cage). Inappropriately treating such data as independent results in imprecise estimates of statistical precision and artificially low *P*-values [44, 45].

Even for individually housed mice, detecting significant differences in food intake is challenging due to high intake variability within and between individuals [46, 47]. Failure to detect statistically significant intake differences is often due to underpowered studies and should not be interpreted as indicating no meaningful intake difference. For example, Fischer *et al.* [48] found a small difference in body fat due to knocking out the FTO gene and claimed that energy intake was not responsible because no statistically significant intake difference was found. However, the energy intake difference necessary to explain the body fat observations with a power of 80% and a significance level of 0.05 would have required a sample size of 4337 per group—compared to the actual sample of around 10. Hence the power to detect a food intake effect was only 5.1% [49].

Individual variation in daily energy intake has a coefficient of variation between 10% and 17% which itself depends on the diet composition [50]. Supplementary Table S2 shows the sample size necessary to detect different effect sizes on food intake in a standard two sample *t*-test with a power of 80% and alpha = 0.05. Given that most studies have sample sizes around 10 per group, the effect size that can be detected between groups is only around 15%–25% difference in daily energy intake. However, a

Table 1 Recommendations for authors, reviewers, and editors of nutritional investigations.

Domain	Commonplace practice	Limitation	Recommendation
Design	Unrefined, grain-based "chows" are commonly utilized as "control" comparators for purified diets	"Chows" and "purified" diets differ substantially in their composition and present an immeasurably confounded design	The use of chows as comparators for purified diets should be limited to pilot investigations and eliminated as the sole source of data for final publication comparisons
	Various "control" and "experimental" purified diet formulations are purchased and utilized across separate experiments that are ultimately juxtaposed in a manuscript	Diet formulations, including those differing in a dietary component of interest, can differ broadly in their overall composition and introduce unquantifiable confounding	Work with commercial diet vendors and trained animal nutritionists before undertaking diet investigations to ensure the proposed study diets are feasible and the appropriate control diets are procured
	An experimental and a control diet are intended to be fed with the aim of targeting a specific metabolic pathway and making causal inferences about an individual ingredient/nutrient in order to inform therapeutic approaches	Diets differ in at least 2 variables, limiting attribution to a single component. Effects may be due to any one variable modified, or the substitution, and such effects may be modified by interacting variables in the background diet. Food components exhibit significant pleiotropy, targeting multiple downstream mechanisms, and rarely exhibit linear dose-response relationships	Employ multi-level ingredient/nutrient substitution matrix designs where possible that consider substitution effects, assess dose-response relationships, as well as effect modification by background diet composition Harmonize diet design with other elements of the experimental design (e.g. genetic, pharmacological manipulations) that can isolate the contributions of potential relevant mechanisms at play. Employ unbiased, high-throughput approaches (e.g. metabolomics) to characterize the metabolic context in which relevant diet-induced mechanisms occur
	Experimental diet manipulations are undertaken with limited feasibility or pilot testing and unclear rigor	Experimental diet manipulations frequently have unintended consequences that compromise the ability to test the intended hypothesis. Confounded attempts may go unpublished and resources wasted, or published and the confounding impacts of the unintended consequences go unrecognized, minimized, or unreported	Undertake pilot studies to confirm expected phenotypes (e.g. weight gain or maintenance), to assess for unintended consequences of dietary manipulations (e.g. food aversion and weight loss, apparent pathology) and to facilitate a priori power analyses
Analysis and reporting		Deviations from protocols or in-tandem decisions can introduce biases and compromise rigor; practices may include selective reporting of assessed outcomes, unjustified and/or nontransparent removal of outliers and other protocol modifications, and reporting of spurious findings as significant	Open pre-registration of studies should become commonplace, including the intended diet formulations, age at diet manipulation, sex of animal, power calculations, all outcomes to be assessed and their method of assessment, and statistical analysis plan
	Diet composition is assumed based on the label compositional analysis	Diet manufacturing and processing, shipment and/or storage conditions can influence diet composition in unintended manners, resulting in alterations to the concentration of compounds of interest being fed. Diet contains numerous unquantified factors that may be relevant effect modifiers for the outcomes studied	Pursue an independent laboratory analysis of commercially purchased diet to ensure expected concentrations of relevant food derived components, especially when conducting long-term studies with >1 lot number. It is advisable that researchers store a frozen aliquot of investigational diets for future analysis and comparison
	Diet information used throughout the study is not specified (e.g. "chow") and/or listed throughout the text. Referenced diets may or may not be open source. Bedding type and consumable enrichment is rarely reported	Without brand and catalog number information, diet composition is challenging to assess. Custom purified diets do not have full diet composition data available through vendor websites, requiring contact with vendors to retrieve such information. Bedding and enrichment can modify metabolism-related phenotypes and cannot be accounted for without reporting	Transparently list the name, formulation, and known composition of all feeds used in the investigation in a main or supplementary table. Bedding type and identifier information should be reported as well as consumable enrichment use
	Total number of rodents is reported per diet group	Rodents are group-housed and diet is delivered at the level of the cage, introducing non-independence of the individual animals. Failing to utilize a cluster analysis of such data results in artificially lower estimates of variance and lower *P* values	Clearly report the unit of randomization within a study (cage or individual rodent) and related relevant parameters (e.g. animals per cluster). Choose the appropriate statistical approach and explicitly justify this in manuscripts

Table 1 Continued

Domain	Commonplace practice	Limitation	Recommendation
	Investigations modifying diet composition do not report longitudinal changes in food intake and body weight. Conclusions about a diet's effects on food intake are made regardless of the statistical power to detect an effect. Diet composition and/or feeding protocols may change the pattern of intake (i.e. duration of fasting between feeding intervals, relation of intake to the circadian cycle)	The impact of diet composition changes on outcomes may be mediated through alterations in energy balance and body composition rather than through independent effects of the diet component. Many studies are underpowered to detect food intake changes that may underlie phenotypes. Few studies assess whether changes in diet composition or feeding protocol imparts their effects through altering the pattern of intake	Report the impact of diet composition modification on longitudinal measures of food intake, body weight, and body composition regardless of whether such variables are the primary outcome of the investigation. Limit conclusions about the impact of diet on components of energy balance when not explicitly powered to do so. Design experiments to manipulate food access to control for alterations in fasting duration and circadian alignment
	A single diet component is highlighted throughout the manuscript	Diets lack a placebo, introducing an inherent relative effect of investigational diets in relation to their selected control. Investigations rarely employ multiple comparators across a dose-response relationship to confidently attribute causal effects to one diet component	Titles, abstracts, and in-text descriptions should transparently report effects of the investigational diet relative to comparator diets, highlight relevant substitution effects, and make evident the degree of confidence in the dose-response relationships. Named diets and their compositions should be clearly detailed in the main manuscript leaving readers with a clear appreciation for variables differing between diets

sustained 3% increase in energy intake, with mice eating on average 65 kJ/d, would lead to a measurable gain in body weight of ~2.2 g based on the relationship of ~0.9 kJ/d per g of body weight [51], and would require a sample size between 176 and 504 mice per group to detect the energy intake difference. Averaging food intake over multiple days is a useful strategy to reduce variation and improve study power [47]. However, diminishing returns are observed beyond about 10 days of averaging (see Supplementary Fig. S2). More data are needed on temporal patterns and variation of intake in group-housed animals to better inform power analyses.

Generalizability and translation

Age, sex, and strain of rodents are influential variables that interact with nutrition and affect the ability to generalize and translate experimental results. For example, nutrient requirements and metabolic responses to diet vary across the lifespan. Because the average age at death of mice is about 800 days while humans live on average about 80 years, the approximate equivalence between mice and humans is that 10 days for a mouse are roughly equivalent to 1 year for a human. However, the key life transition stages do not precisely line up. Mice wean at the age of 3 weeks (equivalent to ~2 years in humans) and complete linear growth around the age of 12 weeks (equivalent to ~8.5 years in humans). Experiments conducted on mice younger than 12 weeks are not readily translatable to adult humans and important differences in experimental outcomes may depend critically on this age difference. For example, Sørensen *et al.* [52] found that protein had a strong leveraging effect on food intake in young mice (aged 9 weeks at diet onset), but this effect was not replicated in mice aged 12 weeks at diet onset [31], potentially because protein requirements are much more important during growth than among adults.

Moreover, feeding behavior is likely influenced by sex in group-housed rodents. Despite common assumptions that female mice will exhibit greater variability due to estrus cycling [53], group housing of males introduces fighting and barbering stresses that in turn introduce variability in intake and energy homeostasis across dominant and subordinate individuals. Individual females, however, are less stressed when group-housed than those kept solitarily [54].

Nutrient requirement estimates are also not tailored to rodent strains, though diet composition-by-strain interactions have been noted for decades when defining the energy, fat, and protein requirements required for growth and reproduction [55]. Recently, such interactions have been observed in a comprehensive metabolic phenotyping of four inbred mouse strains (A/J, B6J, FVB/NJ, NOD/ShiLtJ) fed six diets, including traditional mouse (purified low fat/control, Western) and human diets (American, Mediterranean, Human, Japanese) [56].

Robustness of diet effects in the context of systematically heterogenized experimental variables (e.g. strains, sex, age, and microbiome composition) increases confidence that results are generalizable and potentially translatable to humans. Conversely, the lack of consistently observed effects opens up avenues for understanding novel biological factors that are permissive to certain diet effects, an increasingly emphasized concept in the era of "precision" medicine and nutrition.

Recommendations and conclusions

The complexity of rodent diet intervention studies is challenging to capture in a single brief review, but Table 1 summarizes our major observations about common practices, their limitations, and our recommendations. We have emphasized the important role of diet to elicit widespread physiological changes, so designing and interpreting diet intervention studies should not be inappropriately viewed through a lens of targeting a particular biological mechanism without acknowledging alternative independent mechanisms. Clearly isolating the cause of the diet effects to a single biological mechanism will require multi-omics and quantitative phenotyping approaches, dose-response studies to assess the robustness of the phenotypes, and the incorporation of genetic and pharmacological manipulations to perturb likely causal mechanisms. With these caveats in mind, we believe that carefully designed diet interventions in rodent models hold great promise for both elucidating important metabolic physiology in

health and diseases as well as modulating outcomes in ways that may better translate to humans.

Supplementary data

Supplementary data is available at *Life Metabolism* online.

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Author contributions

All authors collectively drafted and revised the manuscript.

Conflicts of interest

John R. Speakman holds the position of editors-in-chief for *Life Metabolism*, and is blinded from reviewing or making decisions for the manuscript. The other authors declare that no conflict of interest exists.

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