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Protein-leverage in Mice: The Geometry of Macronutrient Balancing and Consequences for Fat Deposition

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Objective: The Protein-Leverage Hypothesis proposes that humans regulate their intake of macronutrients and that protein intake is prioritized over fat and carbohydrate intake, causing excess energy ingestion when diets contain low %protein. Here we test in a model animal, the mouse: (i) the extent to which intakes of protein and carbohydrate are regulated; (ii) if protein intake has priority over carbohydrates so that unbalanced foods low in %protein leads to increased energy intake; and (iii) how such variations in energy intake are converted into growth and storage.
Methods and Procedures: We fed mice one of five isocaloric foods having different protein to carbohydrate composition, or a combination of two of these foods (*N* = 15). Nutrient intake and corresponding growth in lean body mass and lipid mass were measured. Data were analyzed using a geometric approach for analyzing intake of multiple nutrients.
Results: (i) Mice fed different combinations of complementary foods regulated their intake of protein and carbohydrate toward a relatively well-defined intake target. (ii) When mice were offered diets with fixed protein to carbohydrate ratio, they regulated the intake of protein more strongly than carbohydrate. This protein-leverage resulted in higher energy consumption when diets had lower %protein and led to increased lipid storage in mice fed the diet containing the lowest %protein.
Discussion: Although the protein-leverage in mice was less than what has been proposed for humans, energy intakes were clearly higher on diets containing low %protein. This result indicates that tight protein regulation can be responsible for excess energy ingestion and higher fat deposition when the diet contains low %protein.

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INTRODUCTION

There is continuing controversy about how nutrition and especially the macronutrient composition of the diet relates to the development of obesity (1,2). In humans, most emphasis has been placed on the roles of fats and carbohydrates (3,4). Until recently, protein has been largely ignored as a causative factor for obesity, mainly because protein comprises the minor part of the total energy budget and its intake has remained relatively unchanged throughout the development of the obesity epidemic (5).

However, it has recently been suggested that the ratio of protein to non-protein energy in the diet may provide a much stronger predictor of total energy intake than previously thought (5,6). In short-term experiments, protein has been shown to induce higher levels of both satiation and satiety than fat or carbohydrate (e.g., 7,8). Over longer periods, *ad libitum* access to protein-rich diets has been shown to promote and maintain weight loss (9–11), and data suggest that at least part of this effect results from reduced total energy intake (12).

The recently published Protein-Leverage Hypothesis (5) proposes that in humans protein intake is regulated and prioritized over fat and carbohydrate intake in the face of varying ratios of protein to non-protein energy in the diet. As a result, excess energy is ingested on diets containing a low %protein, while an energy deficit is incurred when %protein is high. In both these cases, the absolute intake of protein will remain relatively constant, while intake of fat and carbohydrate will vary substantially.

Here we conduct a detailed study of macronutrient regulation in the mouse—a frequently used model system for the study of human obesity. We have used an experimental design based on state-space geometric models, which were originally derived from studies on insects (13) but in recent years have

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been employed to a range of other animals across the taxa (14), including humans (6). The approach involved three stages. In the first, we test whether mice have the capacity to separately regulate their intake of protein and carbohydrate when fed pairs of nutritionally complementary foods differing in protein to carbohydrate ratio. Second, we measure how strongly mice regulate their intake of protein compared to that of carbohydrate when fed diets containing one of several ratios of protein to carbohydrate, requiring the animals to balance under-eating one macronutrient against overeating the other. Finally, we measured the consequences of these consumption patterns for body composition, notably fat deposition.

METHODS AND PROCEDURES

Animals and housing facilities

One hundred and fifty-two, 5-week-old, outbred male mice of the NMRI strain were purchased from a commercial supplier (Taconic *M & B*—Quality Laboratory Animals and Services for Research). Each animal was housed individually in standard Macrolon cages type 2 (l: 23 cm, w: 17 cm, h: 14 cm) with free access to water. A double layer of Elephant filter (48 × 58 cm^2) was provided as bedding, which was changed twice weekly. Strips of carton (EnviroDri) were provided for nest building. A purpose-designed food-spillage collector was placed in each cage to allow the two foods used in the choice treatments to be kept separated as well as to collect food spillage (**Figure 1**). Food was placed in the groove on each side of the water bottle. In order to eat, mice had to jump into one of the boxes; foods were otherwise out of reach. This prevented the mice from mixing foods and allowed all spillage to be collected in each of the two boxes. In choice treatments, the position of the two foods in the cage was reversed at each feeding time to control for positional effects. Throughout the experiment the mice were maintained at an ambient 24–26 °C and 53–56% relative humidity under a 12:12-h light–dark photoperiod, with lights on at 0800.

Experimental diets

Five semisynthetic test-diets (dry, in pellet form) were specifically designed for the experiment and made by the Altromin GmbH company. Diets were isocaloric and similar in composition except for the ratio of casein to starch (**Table 1**).

Experimental design and procedures

Before the experiment began, mice had an acclimation period of 8 days during which they were kept at experimental conditions but fed *ad libitum* on a standard diet (breeding mice/rat diet Altromin 1310; protein 26.0% dry weight and carbohydrate 55.5% dry weight) suitable for mice at that age (5 weeks). Mice were then given experimental diets (**Table 2**) *ad libitum* for a 32-day period. An additional group of mice (N = 17) were killed at the beginning of the experiment to provide estimates of initial body composition. On a standard diet the expected change in body mass during 32 days, from when the mice are 6 weeks old and until they reach 10 weeks of age, is ~5 g (15).

There were two experiments that were conducted simultaneously. In the first, the aim was to establish whether and to what extent mice regulated their intake of protein and/or carbohydrate. This involved providing them with one of four two-food choice treatments (N = 15, **Table 2**). Perfect regulation of both protein and carbohydrate intake would result in mice adjusting intake of the two foods such that they converge on the same intake trajectory (the intake target) on a protein–carbohydrate plane. In contrast, were they to feed indiscriminately (e.g., by regulating caloric intake independently of nutrient source), they would on average end up eating equal amounts of each food in the choice treatment and hence follow four different intake trajectories. The second experiment aimed to quantify the extent to which mice would balance protein and carbohydrate intake when provided with one of an array of five foods (N = 15, **Table 2**). Specifically, it addressed the question of whether they would maintain protein intake more constant than carbohydrate intake when having to trade one off against the other.

Fresh preweighed portions of food were provided every second day and food remains (including those in the feeding tray and food-spillage collector) were collected and frozen for later weighing. The amount of food given was enough to ensure that animals always had food available, but not to such a degree that weighing errors were exacerbated (16). Fresh food and food remains were dried to constant mass in a

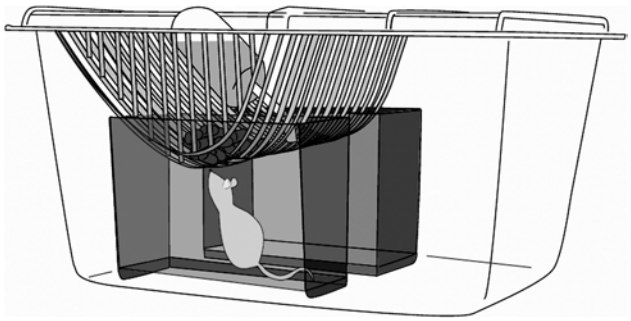

Figure 1 Mice case with mouse and food-spillage collector. The collector was made of plastic and mice could easily crawl up into one of the two boxes, which they had to do in order to eat. This ensured that food placed in different sides of the cage was collected in each box separately. The boxes contained elephant paper at the bottom to absorb water and urine, and the whole collector unit could be removed from the cage to allow easy collection of food remains.

Table 1 Composition of semi synthetic experimental isocaloric diets (g/100 g dry mass)

Diets	P9%	P17%	P23%	P31%	P48%
Crude protein	9.0	16.8	23.2	31.4	47.7
Carbohydrate	71.8	63.9	57.4	49.5	33.1
Crude fat	7.8	7.8	7.8	7.6	7.7
Crude fibre	6.0	6.0	6.1	6.0	6.0
Ash	5.4	5.5	5.5	5.5	5.4
Energy (kcal/g)	3.8	3.8	3.8	3.8	3.8

Diets varied in contents of casein and starch; all other ingredients were kept similar. Values are based on foods' dry weight. Main ingredients: casein, cornstarch, wheat starch, sucrose, sunflower oil, cellulose powder, a mineral mix (K_2CO_3, Coal.Chalk, NaCl, $MgCl_2$, K_2HPO_4, Fe_2O_3, $FeSO_4$, $MnSO_4$, NaF, KI, Na_2SeO_3, Na_2MoO_4, $CoCl_2$, $ZnCO_3$, $CuSO_4$) and a vitamin mixture (C 1,000, Altromin).

Table 2 Experimental design. Mice were fed either one diet (no-choice) or two diets (choice) at the same time.

		Diets				
Treatment groups	*N*	P9%	P17%	P23%	P31%	P48%
No-choice						
P9%	15	X				
P17%	15		X			
P23%	15			X		
P31%	15				X	
P48%	15					X
Choice						
P9% vs. P48%	15	X				X
P9% vs. P31%	15	X			X	
P17% vs. P48%	15		X			X
P23% vs. P48%	15			X		X

vacuum-oven at 50 °C. Food intake was calculated as the dry mass differences between food given and that remaining.

All treatment groups were housed in the same room, and cage positions were randomly changed at each feeding time. Mice were weighed to the nearest 0.1 mg immediately before the start of the experiment and again after they were killed by cervical dislocation at the end of the experiment.

Body composition analysis

The gut contents of dead mice were flushed out with salt water, and the carcasses were dried to constant mass in an oven at 70 °C. Dried carcasses were first ground in a course mill and again in a Retsch Vibratory Mill (Type MM-2). For analysis of body fat in mice, 1 g samples of homogenate were weighed, extracted with petroleum ether, then reweighed to obtain fat content by mass difference (17). Total nitrogen content was analyzed using a dry combustion analyzer (Na2000), and crude protein content was calculated as total nitrogen × 6.25 (18).

Data analysis

Using the group of mice that were killed at the beginning of the experiment (N = 17), we created regression equations between initial wet mass and body fat content. From this equation we could estimate initial body fat and lean dry mass of all experimental mice. Fat deposition and lean dry mass growth were subsequently calculated using these initial estimates of body composition and actual final body composition. Differences in intakes and body compositions were tested using ANOVA and ANCOVA, respectively, and Bartlett's test allowed analysis on untransformed data ($P > 0.05$) in all cases. Dunnett's tests were used as post hoc to test for differences in total energy intake.

RESULTS

Experiment 1: regulation of protein and carbohydrate intake to an intake target

Mean amounts of the two foods eaten in each treatment group are plotted in **Figure 2.** It is evident that mice did not feed indiscriminately, but clearly distinguished between the foods with different protein content. Over the first 8–10 feeding periods (16–20 days), two contrasts emerge in the comparison of mice fed the P9% vs. P48% (**Figure 2a**) and the mice fed the P9% vs. P31% combination (**Figure 2b**). First, animals in both treatments showed an overall higher intake of the high-protein food (P31% or P48%) than of the P9% food. Second, the relative preference for the high-protein food was greater when it contained a more moderate (P31%) than extreme (P48%) proportion of protein. Such an outcome would be expected if the mice were attempting to regulate both protein and carbohydrate intake to mix a diet composition in the region of 25% protein. A similar compensatory response was evident in a comparison of the other two treatments, in which the extreme-high-protein diet (P48%) was paired with more moderate diets containing P17% (**Figure 2c**) or 23% (**Figure 2d**). In both cases the more moderate, low-protein, diet was eaten in higher relative amounts, and the relative preference of the lower-protein food in the pairing rose progressively as its %protein increased. Indeed, the intake of the P48% food dropped to almost nothing when it was paired with the P23% food, again suggesting a preferred diet composition of ~25% protein.

Beyond feeding period 10 (day 20) there was a pronounced shift in intake patterns. In treatments P9% vs. P48% and P9% vs. P31%, intake of the higher-protein food dropped markedly

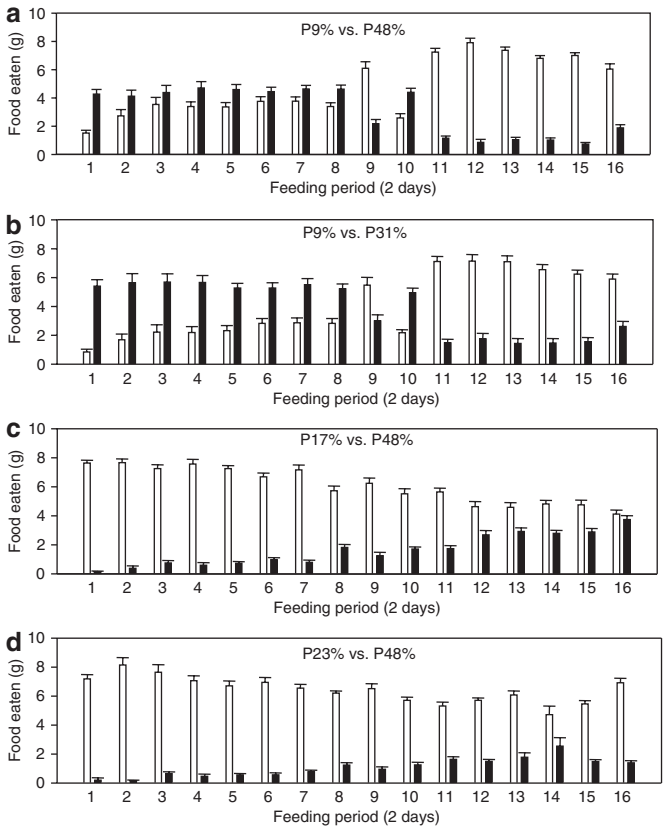

Figure 2 Dry mass intake (g ± s.e.) of the two food types offered under choice conditions. White bars indicate the food type having the lowest %protein, black bars indicate the highest %protein. Intake was measured for 2-day feeding periods over 32 days.

and consumption of the low-protein food rose. This pattern was not apparent in the P17% vs. P48% and P23% vs. P48% treatments (**Figure 2c**,**d**), where there was a steady increase in intake from the higher-protein food and decrease in intake of the lower-protein food across the 32 days, this being more evident in the former treatment.

The outcome of these consumption patterns in terms of macronutrient gains can be seen in **Figure 3**, which plots the cumulative intake of protein and carbohydrate across the 32-day period. The four nutrient intake trajectories converged but not completely, indicating that regulatory responses for protein and carbohydrate, although pronounced, were not perfect. The nutritional rail closest to the predicted intake target was P23%. The distinct vertical inflection at around day 20 (feeding period 10) can be seen for P9% vs. P48% and P9% vs. P31%, leading to mice on the P9% vs. P48% treatment partly converging with those on P17% vs. P48% by the end of the experiment.

Experiment 2: balancing macronutrient intake in no-choice assays

When mice were offered a single food containing one of five ratios of protein to carbohydrate, they were forced to balance overeating one nutrient against under-eating the other, relative to the intake target (i.e., the regulated diet composition from the

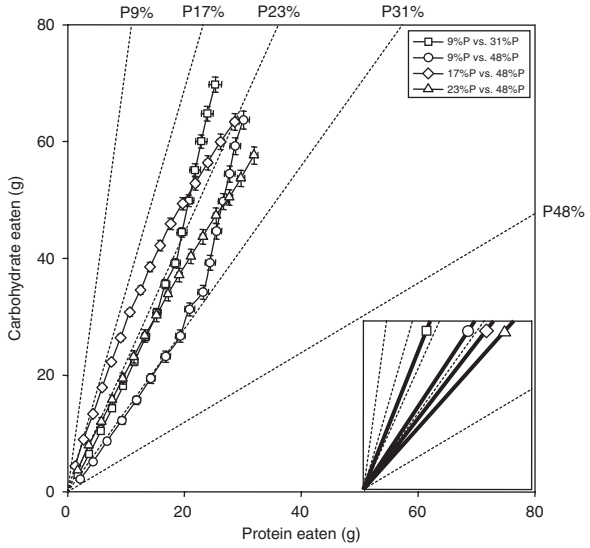

Figure 3 Geometric representation of protein and carbohydrate regulation by mice that were offered a choice between two food types of different protein to carbohydrate ratio. Nutrient intake (gram ± s.e.) was measured for 2-day feeding periods and are illustrated here as cumulated intakes. Thus, the curves show how the trajectories of the total composed diet over time. The dashed lines indicate the trajectories the mice would follow if they only consumed one of the chosen foods, and the bold line in the small box at the lower right corner indicates the trajectories mice would follow if they were not regulating intake at all, thus consuming the two foods at random. Note that the average intakes of the four food combinations (the estimated intake target) throughout the experiment were closest to the rail represented by food P23%.

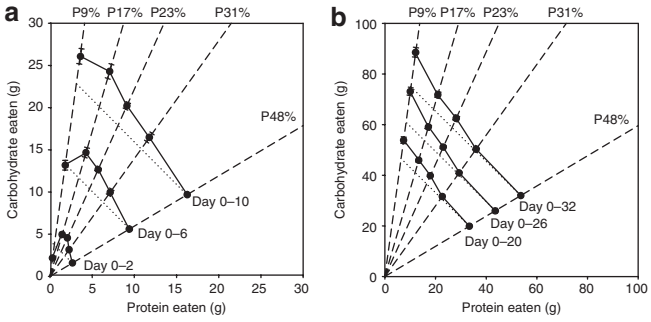

Figure 4 Geometric analysis of protein and carbohydrate intake (gram ± s.e.) when mice were confined to eat only a single food type (no-choice conditions). Dashed lines show the nutritional rails of the five different food types that the mice were confined to follow. Cumulated mean intakes are shown for the days 2, 6, and 10 in (**a**), and 20, 26, and 32 in (**b**). Intake arrays are made by combining the mean intakes and the dotted lines below the intake arrays indicate the –1 slope that the mice would follow if just the total nutrient or energy intake (i.e., the sum of protein and carbohydrate) were regulated. The difference between the intake array and the –1 slope show the degree with which the mice balance the over- or under-consumption of one nutrient against the other.

choice experiment). The outcome of this balancing is plotted in the form of protein–carbohydrate intake arrays in **Figure 4**. If the mice were simply maintaining caloric intake, irrespective of the source of this energy, it would be expected that the intake arrays in **Figure 4** would be straight lines with an inclination of –1 (the sum of protein energy and carbohydrate energy intake

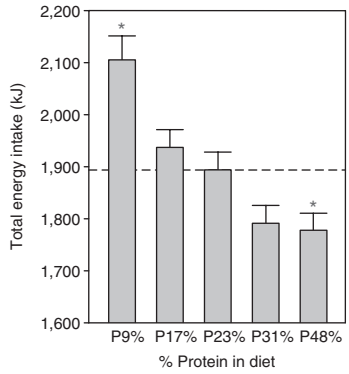

Figure 5 Total energy intake over the full 32 days of the experiment by mice that were fed five different food types under no-choice conditions. The dashed line indicates the energy intake by the mice fed the food closest to the estimated intake target (food P23%). Total energy intake differed significantly between the five different food types (ANOVA, $F_{4,74} = 13.12$, $P < 0.0001$), and the asterisks indicate significant differences from food P23% according to Dunnett's test.

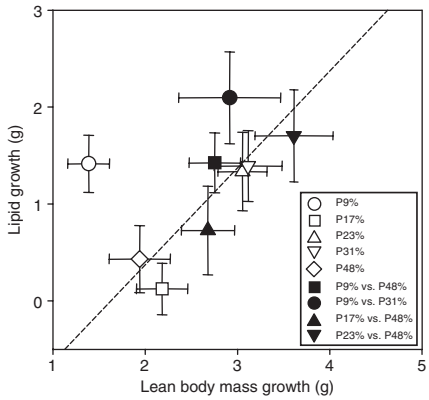

Figure 6 Growth in body lipids and lean body mass (gram ± s.e.) of mice after 32 days on the diets. The dotted line is the regression through the means of all the treatments except for food P9%.

being a constant). This is, however, not what our data reveal. Except for the first week, during which intake of the P9% diet was low (it subsequently recovered), the array assumed a slope that was steeper than –1, demonstrating a stronger regulation of protein intake than of carbohydrate intake. This prioritization of protein intake affected total energy intake (ANOVA, $F_{4,70} = 13.12$, $P < 0.0001$), and the lower the ratio of protein to carbohydrate in the diet, the higher the total energy intake (**Figure 5**). Animals on the diet nearest in composition to the self-selected diet from the choice experiment (P23%) had an intermediate energy intake.

Effects of diet on body composition

Body composition was compared between the nine treatment groups combined across both choice and no-choice experiments (**Figure 6**). When lipid growth was plotted against lean body mass growth, all treatments except for mice confined to the P9% diet formed a single linear relationship (lipid growth = 1.5 × lean mass growth − 1.68 (g), $R^2 = 0.45$; ANCOVA, $F_{1,111} = 82.5$, $P < 0.0001$ for the effect of the covariate, lean body mass

growth on the dependent variable, lipid growth). Within these eight groups, the remaining statistical effect of diet treatment was non-significant (ANCOVA, $F_{7,111} = 2.05$, $P = 0.055$). The data for mice fed P9% diet fell well above the regression through the other eight treatments. These mice, which had the highest energy intake (**Figure 5**), had exceptionally high body fat relative to their low lean growth (**Figure 6**).

DISCUSSION

Our results indicate that mice regulate intake of both protein and carbohydrate if offered the choice between nutritionally complementary foods, but that protein intake is prioritized when the protein to carbohydrate ratio in the diet is fixed. The dominance of protein regulation over total energy intake is not as extreme as has been suggested for humans (5); but nevertheless, our results indicate that mice offer a suitable model for studying protein-leverage effects in human obesity.

Regulation of protein and carbohydrate intake to an intake target

When allowed to feed from two nutritionally complementary foods, the mice selected their diets in a way that was inconsistent with indiscriminate feeding, and the feeding rather appeared to be based on the nutritional composition of the two foods provided. The pattern of regulation was, however, more complex than has been observed for other animals (19–21). Specifically, the intake trajectories of the four different diet combinations did not coincide entirely, as would be the case if the regulatory compensation for macronutrient content of the diet combinations was complete. Although without further experimentation we are unable to account definitively for the divergence between trajectories, some interesting possibilities present themselves. **Figures 2** and **3** show that there was an abrupt change in the pattern of selection of the choice diets around day 20 of the experiment, and it is thus instructive to consider separately the periods preceding and succeeding this point. Over the first period, the intake trajectories of mice given food combinations P9% vs. P31% and P23% vs. P48% converged remarkably, suggesting the precision of regulation that has been observed previously in insects (19,22). In contrast, the two more extreme diet pairings (P9% vs. P48% and, to a lesser extent, P17% vs. P48%) diverged in the direction of higher protein and carbohydrate intake, respectively. The data seem to indicate that the P9% food lacked phagostimulatory power, since the mice that consumed P9% vs. P48% ate relatively more of the P48% food (**Figure 2**) and hence overate protein. On the other hand, when offered in combination with the more moderate P17% food, the P48% food was relatively less stimulatory, resulting in the P17% vs. P48% treatment mice overeating carbohydrate.

Later in the experiment, both the P9% vs. P48% and P17% vs. P48% animals appeared to compensate for the imbalance accrued during the first part, ending up after 32 days at the same point of intake and indeed on the same nutritional rail that the other two groups had followed in the first part of the experiment. An interesting but difficult-to-explain observation is that the other two groups abruptly deviated from this trajectory around day 20. Both groups did so by eating increasing amounts of the P9% food that was largely ignored earlier. Perhaps the observation that in the no-choice experiment the animals fed the P9% food similarly increased their intake of this food in the approach to day 10 is relevant, suggesting the possibility that the increased intake of the low-protein food in both experiments reflected an acclimation period. An alternative explanation for the abrupt shift in diet choice around day 20 may be a dietary response to developmental changes during the experiment. Male rats have been shown to change their preferences toward more carbohydrate-rich diets after puberty (23). In the outbred mouse strain we used in this experiment, males reach puberty at the age between 5 and 8 weeks (15). As our experimental period spanned 6–10½ weeks of age, the increase in carbohydrate intake might have been an adaptation to meet changing nutritional requirements at puberty.

It would be interesting to see whether a prolonged version of this experiment would demonstrate longer-term regulation, in which the results of the two groups containing the P9% food converge on the intake target.

Protein leverage

When the mice were fed single foods, they could not track their changing intake requirements and were forced to balance overeating one macronutrient against under-eating the other. The intake arrays in **Figure 4** show that neither protein nor carbohydrate dominated the intake completely—which would have resulted in the points across the intake array aligning either vertically if protein had complete priority, or horizontally it carbohydrate had been dominant (14). Nevertheless, protein intake was regulated more strongly than carbohydrate intake (**Figure 4**). As a result, mice increased their energy intake as the percentage of protein in the diet declined, and at the end of the experiment there was a 15% difference in energy intake between the diets having highest and lowest protein content (**Figure 5**). Unless such differences in energy intake are counterbalanced by changes in energy expenditure (24), changes in body composition will result. Indeed, mice fed the diet containing the lowest percentage of protein (P9%) ingested the most energy and deposited a disproportionately high amount of fat relative to lean body mass (**Figure 6**). There was a strong relationship between dietary %protein and absolute lipid growth across the remaining diets but the same relationship appeared between %protein and lean body mass (**Figure 6**).

Prioritization of protein intake over other energy sources has been demonstrated in a range of herbivores and omnivores, including insects, birds, and mammals (14), but it need not always occur. Recent experiments conducted on invertebrate and vertebrate predators, which gain a significant proportion of their metabolic energy from protein rather than fat or carbohydrate, indicate that such animals will ingest substantial excesses of protein relative to intake target levels when confined to very-high-protein diets (25,26).

Variation in key nutritional traits could occur not only with previous nutritional history, but also between developmental stages, with maternal nutritional history, and with genotype (14,19,27,28). Such traits might include regulating the ratio and amounts of macronutrients eaten (the intake target, sensu 29,30), a strategy for weighting excesses and deficits of macronutrients when confined to an imbalanced diet (13), and allocation of ingested nutrients to growth and storage (31–33). We have quantified these three traits over 32 days for a single strain of mouse, with all animals sharing a common nutritional history (a 26% protein diet). It is known, or suspected, that mouse strains and genotypes differ in one or more of these traits (34–36), but except for this study, all the three traits have not yet been measured. We hope that our experiments will provide a basis for comparing other mouse strains and mutants used as models in human obesity research, and for exploring effects of maternal nutritional environment on the feeding behavior and metabolic responses of their offspring.

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DISCLOSURE

The authors declared no conflict of interest.

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